Centralspindlin regulates ECT2 and RhoA accumulation at the equatorial cortex during cytokinesis

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Summary

During determination of the cell division plane, an actomyosin contractile ring is induced at the equatorial cell cortex by signals from the mitotic apparatus and contracts to cause cleavage furrow progression. Although the small GTPase RhoA is known to regulate the progression, probably by controlling actin filament assembly and enhancing actomyosin interaction, any involvement of RhoA in division plane determination is unknown. In this study, using a trichloroacetic acid (TCA) fixation protocol we recently developed, we show that RhoA accumulates at the equatorial cortex before furrow initiation and continues to concentrate at the cleavage furrow during cytokinesis. We also demonstrate that both Rho activity and microtubule organization are required for RhoA localization and proper furrowing. Selective disruption of microtubule organization revealed that both astral and central spindle microtubules can recruit RhoA at the equatorial cortex. We find that centralspindlin and ECT2 are required for RhoA localization and furrowing. Centralspindlin is localized both to central spindle microtubules and at the tips of astral microtubules near the equatorial cortex and recruits ECT2. Positional information for division plane determination from microtubules is transmitted to the cell cortex to organize actin cytoskeleton through a mechanism involving these proteins.

Supplementary material available online at
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Introduction

Cytokinesis is the final event in cell division that causes a cell to split into two daughter cells. The division plane in animal cells is determined by microtubules of the mitotic apparatus. The contraction of a ring consisting of actin filaments, myosin II and other proteins induces ingression of the cleavage furrow (Guertin et al., 2002). However, the molecular mechanisms that determine the division plane and assemble the contractile ring remain poorly understood.

The mitotic apparatus consists of three types of microtubule organization: spindle microtubules formed between two spindle poles, astral microtubules emanating radially from spindle poles, and central spindle microtubules, anti-parallel overlapping microtubules formed between sister chromatids after chromosomal segregation. Central spindle microtubules are tightly connected to each other by associated proteins (Guertin et al., 2002; Mollinari et al., 2002). Among these, astral or central spindle microtubules are considered to be important for determination of the cell division plane (Burgess and Chang, 2005).

Disruption of all microtubule organization with colchicine (Hamaguchi, 1975) prevents formation of the cleavage furrow. Rappaport (Rappaport, 1961) in a micromanipulation study of echinoderm eggs originally proposed that astral microtubules determine the cleavage plane and the results from cultured mammalian cells are similar (Rieder et al., 1997). By contrast, several studies of cultured cells or *Drosophila* mutants (Williams et al., 1995; Cao and Wang, 1996; Bonaccorsi et al., 1998) indicate that central spindle microtubules are essential for determination. Other investigators have recently postulated that microtubule dynamics are related to induction of the furrow. In drug-induced monopolar Ptk2 cells, a subpopulation of stable microtubules extending from chromosomes to the cell cortex appear to induce furrow formation (Canman et al., 2003) and the authors proposed a model in which stable microtubules induce furrows under normal conditions. Micromanipulation studies of grasshopper spermatocytes have suggested that microtubule bundles regardless of source induce cleavage furrows (Alsop and Zhang, 2003). 'Microtubule bundles' have been also observed in *Drosophila* spermatocyte cytokinesis: time-lapse imaging revealed that microtubules make cortical contact and then become bundled at the future cleavage site (Inoue et al., 2004). All of these reports presume that 'cleavage signals' on microtubules induce the assembly of actomyosin contractile rings on cell cortices. However, the molecular identity of such signals is unknown.

Small GTPases of the Rho family including Rho (A, B and C), Rac (1, 2 and 3) and Cdc42 regulate the actin cytoskeleton during many cellular events (Etienne-Manneville and Hall, 2002). Generally, GTPases are molecular switches that mediate

subcellular signaling by cycling between active (GTP-bound) and inactive (GDP-bound) forms. Conversion of these two states is regulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). When cells are injected with the Rho-specific inhibitor, *Botulinum* C3 exoenzyme before furrowing or during cytokinesis, furrows do not form or regress with concomitant disruption of the contractile ring in embryos or cultured cells (Kishi et al., 1993; Mabuchi et al., 1993; O'Connell et al., 1999). These data indicate that Rho is required for both the initiation and progression of cytokinesis. Furthermore, upstream and downstream factors of the Rho GTPase are important for cytokinesis. A RhoGEF, human ECT2 and its *Drosophila* orthologue Pebble are required for cytokinesis (Prokopenko et al., 1999; Tatsumoto et al., 1999). *Caenorhabditis elegans* CYK4, human HsCYK4/MgcRacGAP and *Drosophila* RacGAP50C are RhoGAPs and also required for cytokinesis (Jantsch-Plunger et al., 2000; Hirose et al., 2001; Mishima et al., 2002; Somers and Saint, 2003). The Rho targets, ROCK/Rho-kinase and citron kinase are implicated in the regulation of cytokinesis (Madaule et al., 1998; Yasui et al., 1998; Kosako et al., 2002; D'Avino et al., 2004; Echard et al., 2004; Naim et al., 2004; Shandala et al., 2004). These kinases have been reported to increase the level of myosin regulatory light chain phosphorylation (Kawano et al., 1999; Yamashiro et al., 2003). Another Rho target protein, mDia, a mammalian formin, localizes at the cleavage furrow in Swiss 3T3 cells (Watanabe et al., 1997) and regulates actin assembly in the contractile ring (Lee et al., 1999; Pelham and Chang, 2002; Tolliday et al., 2002). Taken together, Rho has been proposed to enhance actomyosin interaction through its target proteins and that regulators of Rho are also involved in cytokinesis. Therefore, it is a reasonable assumption that 'cleavage signals' from microtubule organizations regulate Rho localization and/or activity for induction of the cleavage furrow at the correct location. However, the precise mechanisms of Rho localization and its activation remain unclear.

We recently developed a TCA-fixation protocol and found it useful for Rho staining (Hayashi et al., 1999; Kosako et al., 2002; Yonemura et al., 2004). We detected RhoA accumulation at the equatorial cortex before and during furrowing using this technique. Disrupting the cytoskeleton indicates that RhoA localization in cleavage furrows depends on microtubule organization required for positioning of the division plane. We tested several proteins that might regulate RhoA localization using the RNAi method and found that centralspindlin and ECT2 are essential for RhoA localization and furrow induction.

Results

RhoA accumulates at the equatorial cortex before furrowing depending on its activity and microtubule organizations

To understand the spatial relationship between RhoA localization and the area of furrow initiation, we examined HeLa cells during the cell cycle fixed according to a TCA-fixation protocol (Hayashi et al., 1999; Kosako et al., 2002; Yonemura et al., 2004). During interphase, RhoA was distributed throughout the cytoplasm (Fig. 1, interphase). At the onset of the mitotic phase, RhoA began to localize at the cell cortex (Fig. 1, metaphase). Before furrowing, RhoA accumulated at the equatorial cortex, which is a putative furrow region (Fig. 1, arrowhead), suggesting that RhoA accumulation is involved in formation of the furrow. During telophase, RhoA continued to concentrate at the cleavage furrow and finally localized at the midbody as previously described (Madaule et al., 1998; Nishimura et al., 1998) (Fig. 1, anaphase-late telo.).

To understand how RhoA accumulates, the fluorescence intensity of cortical RhoA at the polar and equatorial regions was measured before and after the onset of anaphase (Fig. 2A-C). The pole and equatorial regions of a cell were defined as shown in Fig. 2B. At the equatorial cortex, fluorescence intensity of RhoA increased after the onset of anaphase but the polar region did not significantly change (Fig. 2C). These results indicate that the total amount of RhoA on the cell cortex increases after anaphase. Therefore, RhoA at the cell cortex was probably recruited from the cytoplasm.

To determine whether RhoA localization at the equatorial cortex is regulated by Rho activity or not, a Rho-specific inhibitor, *botulinum* C3 exoenzyme or RhoGDI (Rho GDP dissociation inhibitor) that functions as an endogenous Rho inhibitor by binding to Rho in both an active and inactive forms and preventing interactions with effector targets (DerMardirossian and Bokoch, 2005) was injected into HeLa cells before cytokinesis. These cells did not show furrowing as reported elsewhere (Kishi et al., 1993; Mabuchi et al., 1993;

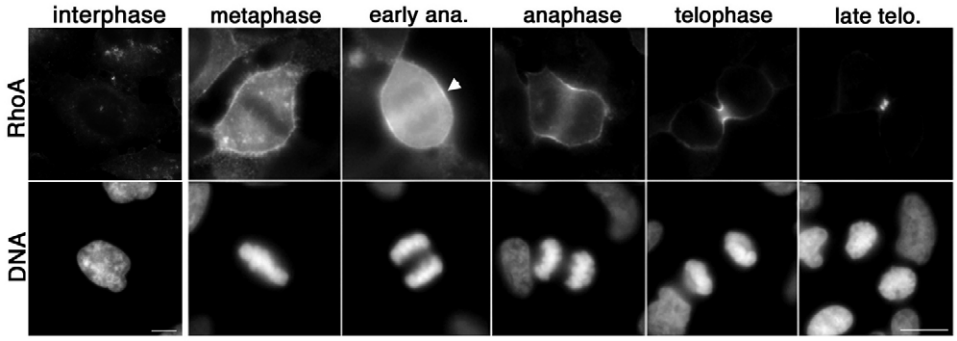
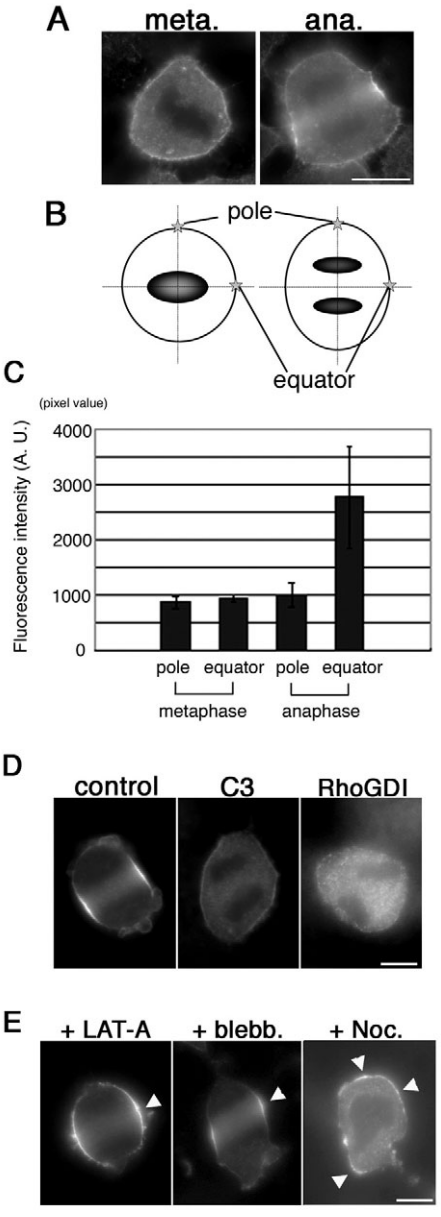


Fig. 1. Localization of RhoA and DNA during cytokinesis in HeLa cells. In interphase, RhoA (upper panels) distributes diffusely in the cytoplasm. After onset of mitosis, RhoA begins to accumulate at the cell cortex (metaphase). Accumulation of RhoA at the equatorial cortex is detected before furrowing (arrowhead) and after which it forms a ring structure at the cleavage furrow (anaphase to telophase). Finally, RhoA localizes to the midbody (late telo.). Lower panels show DNA staining. Bar, 10 μm.

O'Connell et al., 1999). Both C3-inactivated RhoA and inactivated RhoA associated with RhoGDI failed to accumulate at the equatorial cortex (Fig. 2D) [100% of C3-injected cells (n=17); 70% of RhoGDI-injected cells (n=10)], indicating that RhoA activity is required for its localization at the equatorial cortex.

Since RhoA regulates the actin cytoskeleton during cytokinesis (Mabuchi et al., 1993; Drechsel et al., 1997; O'Connell et al., 1999; Tolliday et al., 2002) and because microtubules in the mitotic apparatus are required for division plane determination (Burgess and Chang, 2005), we tested the involvement of both the filamentous actin (F-actin) and microtubule cytoskeletal components in RhoA accumulation at the equatorial cortex. Although disruption of F-actin by latrunculin A or inhibition of myosin II ATPase activity by blebbistatin inhibited furrowing, they did not affect RhoA localization (Fig. 2E, arrowhead; 100% of treated cells in both cases, n=10 for LAT-A and n=12 for blebbistatin). To facilitate the monitoring of microtubules in living cells treated with nocodazole, we used *Xenopus* A6 cells expressing *Xenopus* β-tubulin-GFP. Microtubule organization in A6 cells was disrupted quickly with nocodazole applied immediately after separation of the chromosomes (data not shown). In cells where the disruption of microtubule organization was confirmed by β-tubulin-GFP images, cytokinesis failed and RhoA did not correctly accumulate at the equatorial cortex, resulting in a random distribution throughout the cell cortex (Fig. 2E, arrowheads in +Noc. panel; 60% of cells, n=15). Nocodazole was also applied at initiation of furrowing when RhoA is already concentrated at the equatorial cortex (Fig. 1). In this case, the cleavage furrow formed at the equatorial cortex but repeated progression and regression, resulting in failure in the completion of cytokinesis (Fig. 3 and Movie 1 in supplementary material). Furthermore, the position of the furrow was also unstable. Cells fixed after 30 minutes of nocodazole application showed that RhoA accumulation at the equatorial cortex was lost and that RhoA was detected at several sites on the cortex other than equatorial region (Fig. 3B, arrowheads), indicating that microtubule organization is also required for both proper furrowing and the maintenance of RhoA localization at the equatorial cortex. Thus, accurate RhoA localization at the equatorial cortex throughout cytokinesis depends not on the actomyosin cytoskeleton but on proper microtubule organization.



Both astral and central spindle microtubules can recruit RhoA to the equatorial cortex

Central spindle or astral microtubules in the mitotic apparatus are considered to be important for determination of the cell division plane (see Introduction). To determine which type of microtubule organization regulates RhoA localization at the equatorial cortex, we disrupted astral microtubules or central spindle microtubules selectively.

First, astral microtubules were selectively disrupted using a low dose of nocodazole in NRK-52E cells (O'Connell and Wang, 2000). These cells are flat and appropriate for

Fig. 2. RhoA activity and microtubule organization are required for RhoA localization at the equatorial cortex. (A-C) Quantification of RhoA density at the equatorial cortex during metaphase or anaphase in HeLa cells. (A) Typical RhoA immunofluorescence images at metaphase (meta.) and anaphase (ana.). (B) Schematic drawing of cells used to measure RhoA fluorescence intensity. Stars represent points for measuring RhoA density at the cell pole and the equator. Dark ovals represent nuclei. (C) Fluorescence intensities of RhoA at the pole and the equator. RhoA density at the equator significantly increases after metaphase (n=10). (D) RhoA activity is required for localization at the equatorial cortex. HeLa cells were injected with *Botulinum* C3 exoenzyme (C3), RhoGDI or fluorescein dextran (control) before metaphase and incubated until control cells entered anaphase. No RhoA accumulation is evident in C3- or RhoGDI-injected cells. (E) Microtubule organization is required for RhoA localization at the equatorial cortex during cytokinesis. HeLa cells were treated with 200 μM latrunculin A or with 100 μM blebbistatin before metaphase onset and incubated until control cells entered anaphase. RhoA normally accumulated at the equatorial cortex (+LAT-A, + blebb., arrowheads). A6 cells expressing β-tubulin-GFP were incubated with 16 μM nocodazole just after anaphase onset for 30 minutes. RhoA did not localize at the equatorial cortex but scattered on the cortex (+ Noc., arrowheads). Bars, 10 μm.

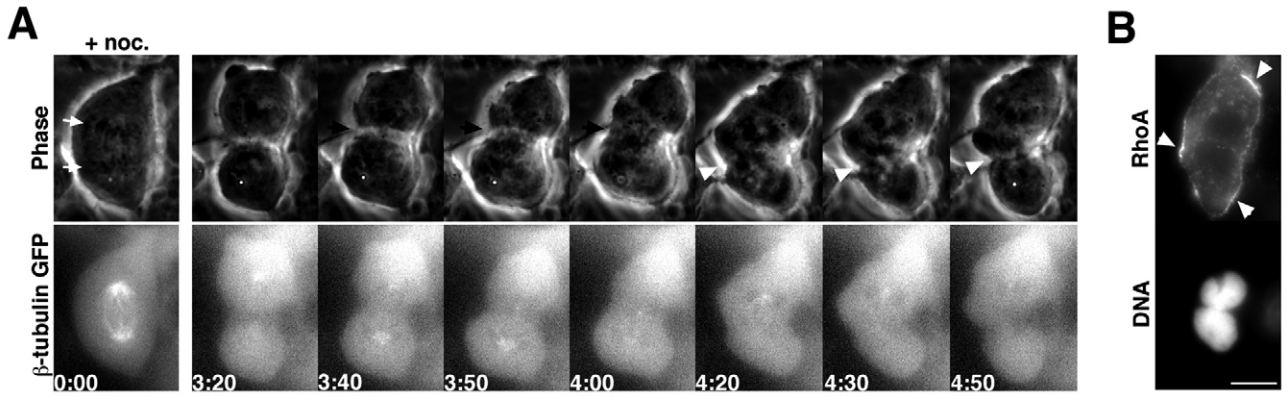


Fig. 3. Microtubule organization is required for stable furrow progression and maintenance of RhoA localization. (A) Selected frames of Movie 1 in supplementary material showing phase-contrast images (upper panel) and β-tubulin images (lower panel) of A6 cells expressing β-tubulin-GFP. At initiation of furrowing when RhoA accumulated at the equatorial cortex, 16 μM nocodazole was added to the medium. Chromosomes are indicated by white arrows. A furrow (black arrows) formed and ingressed (3:20). But it quickly regressed (3:40-4:00). Then another furrow (white arrowheads) formed at a different position and ingressed (4:20-4:50). The furrow also regressed. This cell did not complete cytokinesis within 30 minutes. The elapsed time (minutes and seconds) from nocodazole treatment is indicated at the lower left corner of each panel. (B) The same cell as in A was fixed at 30 minutes of nocodazole application and stained for RhoA and DNA. RhoA localization was not restricted to the equatorial cortex but was found at several sites on the cortex (upper panel, arrowheads). Failure in cytokinesis is revealed by the formation of two nuclei (see DNA in lower panel). Bar, 10 μm.

observation of the relationship between microtubules and the cell cortex. In cells treated with low-dose nocodazole, cytokinesis failed and they became binucleate with no signs of furrowing. RhoA did not localize at the cell periphery where astral microtubules were almost lost (Fig. 4A,B). By contrast, RhoA specifically accumulated at the equatorial cortex near the cell center close to the central spindle microtubules (Fig. 4A,B). Measurements of RhoA fluorescence intensity revealed that RhoA accumulation at the central region of the cell was similar between control and nocodazole-treated cells (Fig. 4B). As vertical images (Fig. 4A, images 1, 2 and 4, red arrows) show, RhoA accumulated at both dorsal and ventral sides of the cortex, but not significantly at the cortex near the periphery of the cells exposed to nocodazole (Fig. 4A, image 3). Along the area of RhoA accumulation of nocodazole-treated cells, actin filaments and activated myosin II, revealed by staining of phosphorylated myosin light chain, also accumulated at the cortex (Fig. 4C), indicating that signals for cleavage furrow induction were simultaneously transmitted from central spindle microtubules to the cortex. These results show that astral microtubules are essential for localizing RhoA and inducing contractile ring formation at the equatorial cortex of the cell periphery in these cells. In addition, central spindle microtubules also recruit RhoA to the cortex when the central spindle microtubules are close enough to the cortex (Fig. 4A) because total disruption of microtubules affected RhoA localization (Fig. 2E).

Next, we disrupted organization of central spindle microtubules by depletion of PRC1. PRC1 is a microtubule-bundling protein and maintains organization of central spindle microtubules as anti-parallel microtubule bundles (Mollinari et al., 2002). We used human HeLa cells for depletion of several proteins including PRC1 by RNAi in this study because human cDNA sequences for the proteins are known and because RNAi is highly potent for silencing genes with this cell line. PRC1-depleted HeLa cells showed selective disruption of central spindle microtubules whereas intact astral microtubules were maintained (Fig. 4D, upper panels). As in control cells, RhoA was detected normally at the equatorial cortex in PRC1-depleted cells (71.92% of PRC1-depleted cells, n=57; Fig. 4D, arrowheads). Furrowing was often obvious at the equatorial region where RhoA accumulated, however, cytokinesis was incomplete in the PRC1-depleted cells as described elsewhere (Mollinari et al., 2002). Therefore, organization of central spindle microtubules is dispensable for RhoA accumulation at the initiation of furrowing in HeLa cells. Instead, astral microtubules appear to be responsible for RhoA accumulation at the equatorial cortex. These data indicate that both astral and central spindle microtubule organization have the ability to recruit RhoA close to the cortex and contribute to contractile ring formation.

ECT2, HsCYK4 and MKLP1 are key regulators for RhoA localization

To elucidate the molecular mechanism of RhoA localization at the equatorial cortex, we tested several candidate proteins that could connect microtubules and RhoA activity. Candidate proteins may localize to both astral and central spindle microtubules because both types of microtubule organization induced RhoA accumulation at the equatorial cortex (Fig. 2E). They also may regulate RhoA activity because the activity is required for RhoA localization at the cortex (Fig. 2D).

Among the candidates, we found that ECT2 (RhoGEF), HsCYK4/MgcRacGAP (RhoGAP), and MKLP1/CHO1, a kinesin that form a complex with HsCYK4 named centralspindlin (Mishima et al., 2002), are key regulators. They reportedly localize to microtubule organizations and are important for cytokinesis (Prokopenko et al., 1999; Tatsumoto et al., 1999; Jantsch-Plunger et al., 2000; Hirose et al., 2001; Matuliene and Kuriyama, 2002; Mishima et al., 2002; Somers and Saint, 2003), although the precise role of each molecule in cytokinesis is unclear. To determine whether this complex is

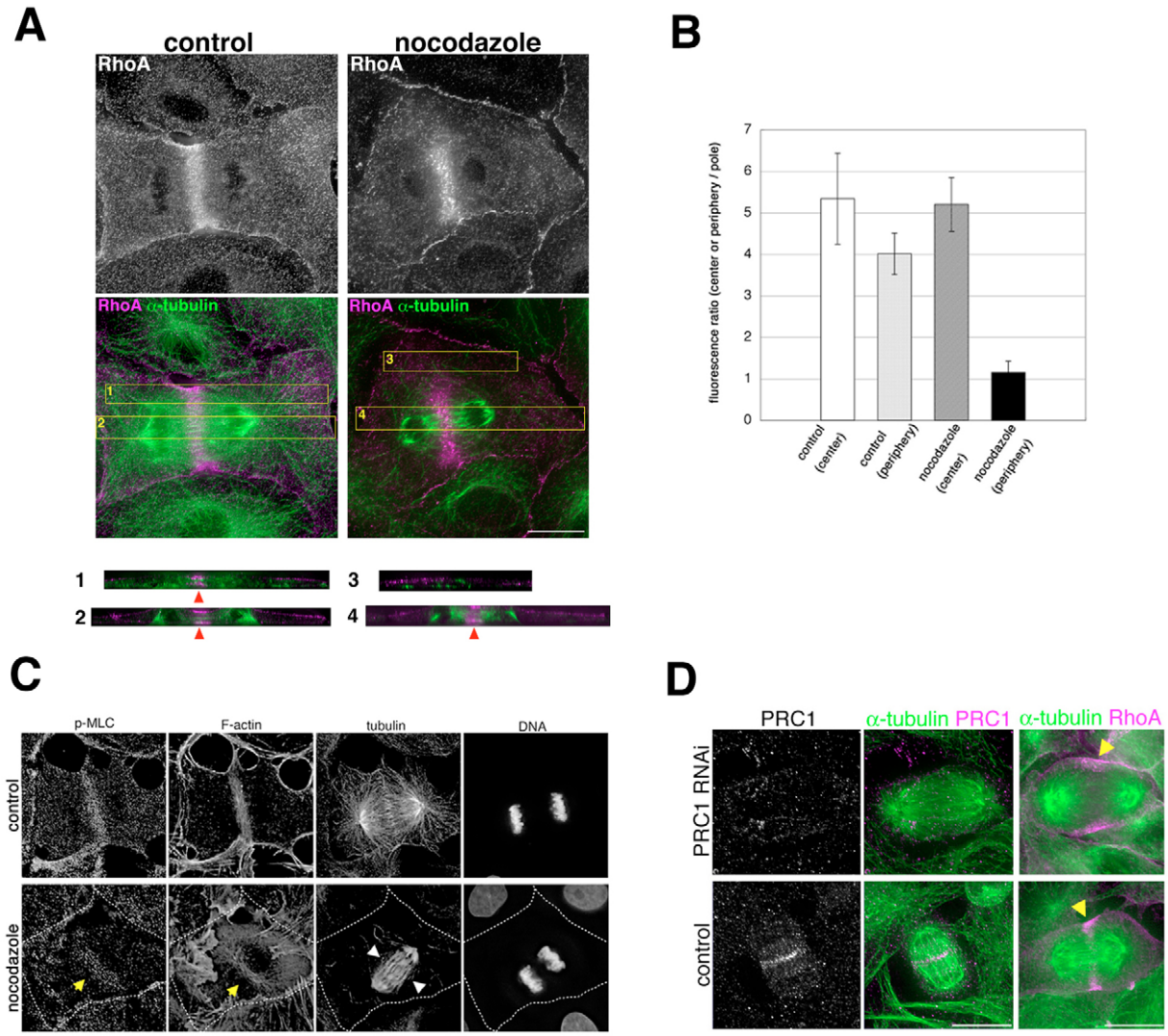
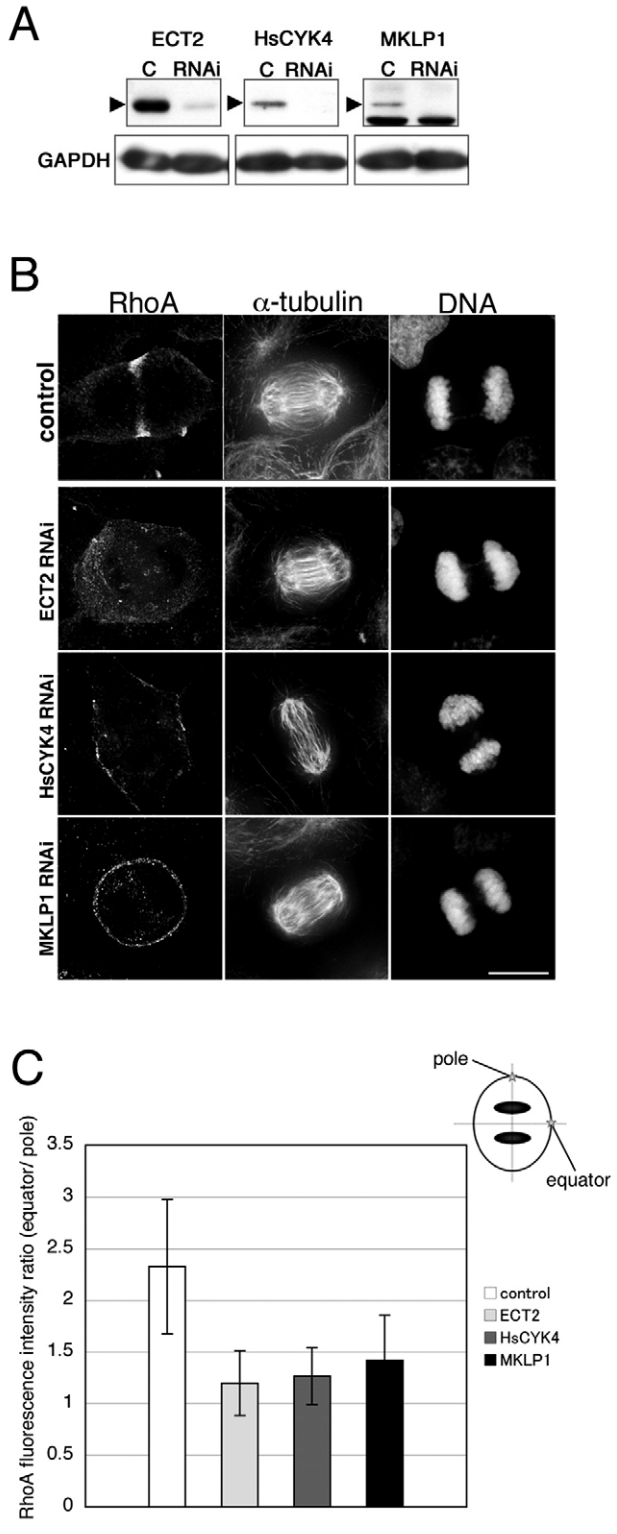


Fig. 4. Astral and central spindle microtubules can localize RhoA at the cell cortex. (A) Control and 150 nM nocodazole-treated NRK-52E cells at anaphase stained for RhoA (upper panels and magenta in lower panels and images 1-4) and α-tubulin (green in lower panels and images 1-4). Upper and lower panels, *X-Y* (horizontal) images of cells projected to one plane from serial optical sections. Images 1-4 represent *X-Z* (vertical) images projected to one plane from serial reconstructed sections from the squares highlighted in the lower images, showing cortical localization of RhoA. Red arrowheads indicate cell equators. In cells treated with this dose of nocodazole, astral microtubules were selectively disrupted. RhoA accumulation is not evident at the peripheral equatorial cortex without astral microtubules (image 3) but is clear around the cell center near central spindle microtubules (image 4). (B) Quantification of RhoA accumulation in control and low-dose nocodazole-treated cells. RhoA accumulation is expressed as ratio of fluorescence intensity at the equatorial cortex in the cell center (white and dark gray bars) or in the cell periphery (light gray and black bars) over the fluorescence intensity at the polar cortex. In nocodazole-treated cells, RhoA accumulation is significantly reduced at the equatorial cortex near the cell periphery (black bar). Values are means ± s.e.m. of three images. (C) Central spindle microtubules induce partial contractile ring formation in nocodazole-treated NRK-52E cells. Phosphorylated myosin light chain (p-MLC) and F-actin are accumulated (lower panels, yellow arrows) along the equatorial cortex at the cell center close to central spindle microtubules (arrowheads in tubulin panels) in nocodazole-treated cells. DNA was also stained. Dotted lines in lower images indicate cell peripheries. (D) RhoA localization is normal in cells with disrupted central spindle microtubules. Left panels, PRC1 staining in HeLa cells. Middle panels, merged images with PRC1 (magenta) and α-tubulin (green). Right panels, merged images of RhoA (magenta) and α-tubulin (green). PRC1 is localized at central spindle microtubules in control HeLa cells. PRC1 depletion (PRC1 RNAi) resulted in disruption of central spindle microtubules. RhoA accumulation (yellow arrowheads) was normal in PRC1-depleted cells. All images in C and D are *X-Y* images projected to one plane from serial optical sections. Bars, 15 μm.

required for RhoA localization, HeLa cells were depleted of ECT2, HsCYK4 or MKLP1 by RNAi (Fig. 5A). In HsCYK4- or MKLP1-depleted cells, central spindle microtubules were aberrant as described (Mishima et al., 2002), whereas ECT2-depleted cells have a normal microtubule organization (Fig. 5B). In all cases, RhoA accumulation at the equatorial cortex was reduced. In cells with no RhoA accumulation at the equatorial cortex during telophase judged from DNA images, no furrow was observed (Fig. 5B), suggesting that these proteins are indispensable for initiation of furrowing. Time-

lapse recordings revealed that initiation of furrowing was sometimes greatly impaired in ECT2-, HsCYK4- and MKLP-depleted cells (Movies 2-4 in supplementary material). We measured RhoA fluorescence intensity and calculated the ratio of intensity at the equatorial cortex to at the polar cortex (Fig. 5C) demonstrating that MKLP1, HsCYK4 and ECT2 are required for RhoA localization at the equatorial cortex to form a contractile ring.



To reveal the spatial relationship between this complex and RhoA, we carefully examined the localization of these proteins. ECT2 was detected in the central spindle microtubules, the cleavage furrow and throughout the cytoplasm (Tatsumoto et al., 1999) (Fig. 7A). As for localization of centralspindlin, new information was obtained: before furrowing, HsCYK4 and MKLP1 localized not only to the central spindle microtubules as described (Matuliene and Kuriyama, 2002; Mishima et al., 2002), but also at the tips of astral microtubules near the equatorial cortex (Fig. 6A,B, arrows). Although ECT2-depletion did not affect HsCYK4 and MKLP1 localization to both the central spindle microtubules and tips of astral microtubules (Fig. 7A, ECT2 RNAi, arrows), HsCYK4 or MKLP1 depletion affected ECT2 localization both at the equatorial cortex and the central spindle microtubules (Fig. 7A, arrows in lower panels). In addition, MKLP1 did not localize to microtubules in HsCYK4-depleted cells and vice versa (data not shown) probably because they need to form a complex for their localization in HeLa cells (Mishima et al., 2002). These results suggest that centralspindlin localized at the tips of astral microtubules can induce RhoA accumulation at the equatorial cortex through recruitment of ECT2. Recently, Mollinari et al. (Mollinari et al., 2005) reported that although in the absence of PRC1 the centralspindlin components were absent from the anaphase spindle, initiation of furrowing was not affected. We carefully compared the distributions of the centralspindlin and RhoA in the PRC1-depleted cells. Centralspindlin dispersed from the central spindle microtubules as reported but not disappear from the cell equator and accumulated at the equatorial cortex with RhoA (Fig. S1 in supplementary material). These observations in the PRC1-depeleted cells also support our idea that localization of centralspindlin determines RhoA localization at the equatorial cortex.

To further confirm the interaction of centralspindlin and ECT2 at the molecular level, we performed immunoprecipitation experiments. Extracts of HeLa cells synchronized at anaphase-telophase were immunoprecipitated with anti-MKLP1, anti-HsCYK4 and anti-ECT2 antibodies. Association of MKLP1 and HsCYK4 as centralspindlin

Fig. 5. ECT2, HsCYK4 and MKLP1 are required for RhoA localization during cytokinesis. (A) Depletion of ECT2, HsCYK4 and MKLP1 was confirmed by immunoblotting with each antibody. Control HeLa cells treated with a scrambled siRNA (C) and cells depleted of each protein by RNAi (RNAi). GAPDH was detected as a loading control. (B) RhoA disappears from the equatorial cortex during cytokinesis in cells depleted of ECT2 (ECT2 RNAi), HsCYK4 (HsCYK4 RNAi) or MKLP1 (MKLP1 RNAi). Cells were stained with antibodies to RhoA and α-tubulin and DAPI. All images are *X-Y* images of cells projected to one plane from serial optical sections. (C) Quantification of RhoA accumulation in control and ECT2-, HsCYK4-, and MKLP1-depleted HeLa cells. RhoA accumulation is expressed as ratio of fluorescence intensity at the equatorial cortex over the fluorescence intensity at the polar cortex. Stars on the schematic drawing of cells (right) represent points for measuring RhoA intensity at the cell pole and the equator. In ECT2 (light grey bar; n=35), HsCYK4 (dark grey bar; n=36) and MKLP1 (black bar; n=39) siRNA-treated cells, RhoA accumulation is significantly reduced at the equatorial cortex compared with that in cells treated with scrambled siRNA (control; white bar; n=20). Bar, 15 μm.

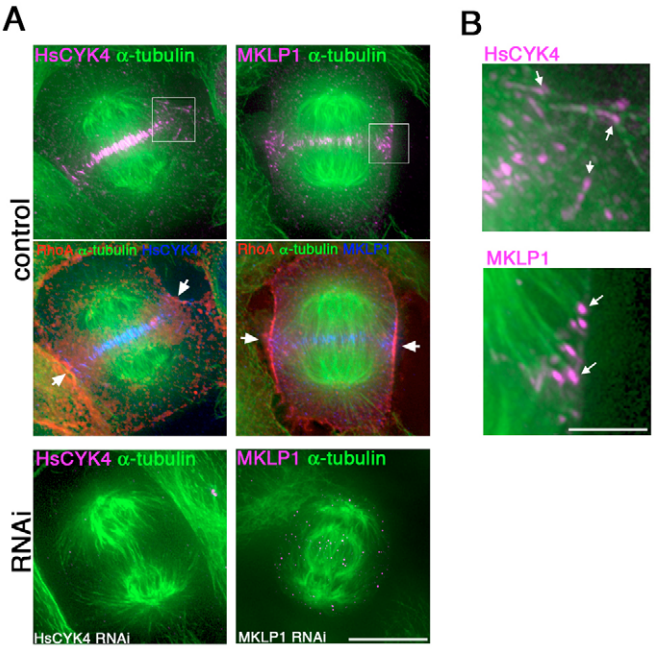


Fig. 6. Localization of HsCYK4 and MKLP1 in HeLa cells. (A) HsCYK4 and MKLP1 localize to central spindle microtubules and the tips of astral microtubules in control cells (control) but not in siRNA-treated cells (RNAi). RhoA (red, in middle panels) accumulated at the equatorial cortex (arrows) in control cells. (B) Magnified images of the boxed areas of A. Arrows indicate tips of the astral microtubule (green) with accumulated HsCYK4 or MKLP1 (magenta). All images in A and B are *X-Y* images projected to one plane from serial optical sections. Bars, 15 μm (A); 5 μm (B).

(Mishima et al., 2002) was confirmed (Fig. 7B). We further found that ECT2 binds to centralspindlin (Fig. 7B). These molecular interactions may be involved in the mechanism of RhoA accumulation at the equatorial cortex by centralspindlin through recruitment of ECT2.

Discussion

Behavior of RhoA at cleavage furrows during cytokinesis

Rho reportedly accumulates after furrowing at the cleavage furrow and then remains at the midbody (Takaishi et al., 1995; Nishimura et al., 1998; Yoshizaki et al., 2003). Recent FRET (fluorescence resonance energy transfer) data have shown that RhoA activity does not increase at the equatorial cortex during early anaphase and that it starts to increase during telophase (Yoshizaki et al., 2003). Biochemical pull-down assays of RhoA activity also indicate that the level of GTP-Rho is maximal during telophase (Kimura et al., 2000). However, this study indicates that active RhoA accumulates at the equatorial cortex before furrowing (Fig. 1). This discrepancy might be due to methodological differences. The TCA-fixation protocol is suitable for detection of RhoA localization, and immunofluorescence microscopy should have allowed detection of small and local changes in the state of endogenous Rho. It is reasonable for RhoA to localize at the equatorial cortex before furrowing, because inhibition of RhoA activity or localization prevents furrow induction.

Measurements of the fluorescence intensity of RhoA at the equatorial cortex revealed that RhoA is recruited directly from

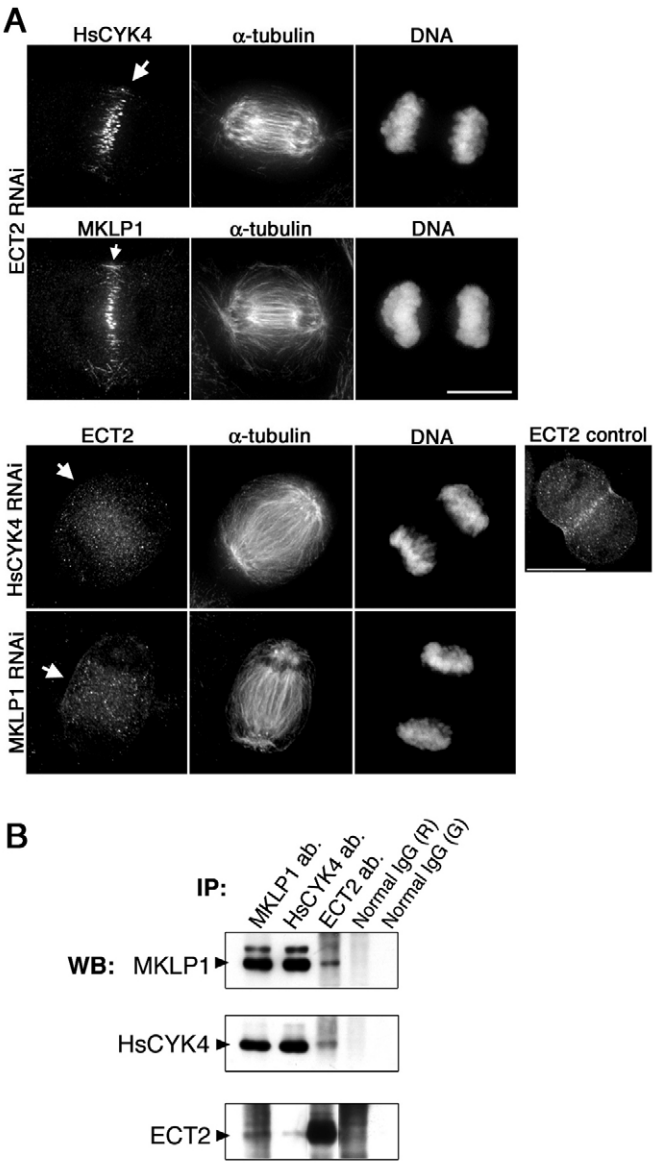


Fig. 7. Relationship between centralspindlin (HsCYK4/MKLP1) and ECT2. (A) HsCYK4 and MKLP1 regulate localization of ECT2 during cytokinesis. Localization of HsCYK4 and MKLP1 was not affected by ECT2 depletion (ECT2 RNAi, arrows). Compared with control cells (ECT2 control), ECT2 localization was lost in HsCYK4- or MKLP1-depleted cells (HsCYK4 RNAi, MKLP1 RNAi, arrows). All images are *X-Y* images projected to one plane from serial optical sections. (B) ECT2 forms a complex with MKLP1 and HsCYK4 in HeLa cells. As indicated above each lane, cell lysates were immunoprecipitated with anti-MKLP1, anti-HsCYK4 or anti-ECT2 antibodies. As a control, rabbit (R) and goat (G) IgG was used. Immunoprecipitates were then analyzed by western blotting with antibodies represented at the left side of each panel. MKLP1, HsCYK4 and ECT2 were detected in the immunoprecipitates of anti-MKLP1, anti-HsCYK4 and anti- ECT2 (black arrowheads), indicating that these proteins form a complex in vivo. Bar, 15 μm.

the cytoplasm to the equatorial cortex at early anaphase because cortical actomyosin contraction force was not required for RhoA localization (Fig. 2E) and RhoA fluorescence intensity increased only at the equatorial cortex compared with

the polar region of the cell cortex (Fig. 2C). Recently we demonstrated that C3 injected into MDCK II epithelial cells caused no change in RhoA localization at the lateral membrane (Yonemura et al., 2004). We speculate that the mechanism of RhoA accumulation at plasma membranes in mitotic cells is different from that in interphase cells. RhoA accumulates at the cleavage furrow not only through the insertion of its C-terminal lipid to the membrane but also through interactions of RhoA with other molecules which are affected by ADP ribosylation of RhoA catalyzed by C3.

We demonstrate that RhoA accumulation at the equatorial cortex depends on microtubule organizations rather than actomyosin contraction force (Fig. 2E and Fig. 3). They are required for proper RhoA localization not only at the initiation of cytokinesis but also during progression of cytokinesis (Fig. 3). Although it is reported that microtubule depolymerization results in the activation of Rho in interphase cells (Ren et al., 1999), the role of microtubules in Rho localization has not been addressed. This study provides the first evidence showing that RhoA localization is regulated by microtubules. Furthermore, considering the behavior of RhoA at the cleavage furrow, RhoA accumulation at the cortex reflects the transmission of signals for division plane determination from the mitotic apparatus to the cortex. We think that RhoA accumulation can be used as an index of signaling for division plane determination. This would be informative especially when cells do not form visible furrows but signals are locally transmitted (Fig. 4A).

The role of accumulation of RhoA at the equatorial cortex

Although RhoA is essential for cytokinesis in embryos and in cultured cells such as HeLa (Kishi et al., 1993; Mabuchi et al., 1993; O'Connell et al., 1999), microinjected C3 does not prevent cytokinesis in some adherent types of cultured cells (O'Connell et al., 1999; Yoshizaki et al., 2004). In addition, inhibiting the Rho upstream factors, ECT2 and HsCYK4, only slightly disturbs cytokinesis in Rat1 cells (Yoshizaki et al., 2004). We confirmed that when NRK-52E cells were injected with C3, cytokinesis occurred without RhoA localization at the cell equatorial cortex (data not shown). However, the shape of the cell periphery and the cleavage furrow were abnormal in these cells and cytokinesis sometimes failed to complete (data not shown) (O'Connell et al., 1999).

Furthermore, myosin-null *Dictyostelium discoideum* cells can divide when cells adhere to the substrate but cannot divide in suspension culture (Neujahr et al., 1997; Zang et al., 1997). Although a cleavage furrow formed following nuclear division in a cell-cycle-coupled manner on a substrate, the length of the cleavage process in myosin-null cells was double that in wild-type cells and sometimes the furrows in the former were not centered, resulting in daughter cells of unequal size (Weber et al., 2000). NRK-52E cells adhering to substrate formed furrows in the presence of myosin II inhibitors (Kanada et al., 2005). Such cells attached to a substrate appear to divide by a polar protrusion force in opposite directions. Considering these data, Rho is not essential for cytokinesis in adherent cells, but is prerequisite for the formation and progression of a cleavage furrow at the right time and place by organizing actomyosin. Cells appear to have a primary inefficient mechanism for cytokinesis that is independent of actomyosin contraction.

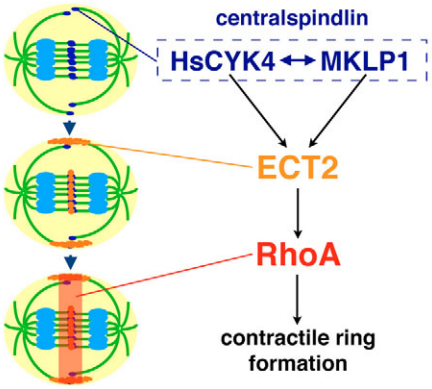


Fig. 8. A model of RhoA localization at the equatorial cortex. HsCYK4 and MKLP1 (centralspindlin) localize both at the tips of astral microtubules towards the equator and at central spindle microtubules and recruit ECT2 at the equatorial cortex and central spindle microtubules. ECT2 then induces RhoA accumulation by elevating RhoA activity.

However, the primary mechanism cannot ensure the accuracy of cytokinesis in terms of the timing and position and formation of a contractile ring required for the serially ordered cell divisions seen in embryos during development where cells do not tightly adhere to the substrate. Cells could have acquired cytokinetic accuracy through the microtubule-dependent Rho accumulation system revealed in the present study.

A model for localization of RhoA at the equatorial cortex during cytokinesis

We propose a model for the molecular mechanism of RhoA localization at the equatorial cortex during the initiation of furrowing (Fig. 8). Centralspindlin localizes both at the tips of astral microtubules and to the central spindle microtubules to localize ECT2 to the equatorial cortex and the central spindle microtubules. ECT2 activates RhoA to accumulate there, resulting in myosin II activation and/or actin polymerization at the cortex to form a contractile ring. RhoA continues to accumulate at the equatorial cortex during the progression of furrowing because centralspindlin-ECT2 complexes at the central spindle microtubules keep contact with the furrow and maintain RhoA activity. In this model, both astral and central spindle microtubules can transmit signals for accumulation of RhoA to the cortex through common molecules including centralspindlin and ECT2. The size of cells compared with that of mitotic spindles appears to determine which microtubule organization plays a major role in several model organisms. In grasshopper spermatocytes, a cleavage furrow can be induced by microtubule bundles, regardless of source (Alsop and Zhang, 2003). It is intriguing to examine whether centralspindlin and ECT2 are also involved in such an experimental system.

The complex consisting of *Drosophila* orthologues of ECT2, HsCYK4 and MKLP1 (Pebble, RacGAP50C and Pavarotti, respectively) has been already identified (Somers and Saint, 2003). From functional analyses of these components, this complex has been proposed as a furrow-inducing signal leading to Rho activation and a model quite similar to ours has already been presented (Saint and Somers, 2003; Somers and

Saint, 2003). No evidence, however, has been provided regarding the regulation of Rho localization by this complex in division plane determination, nor has the component of the complex responsible for its localization been identified. Furthermore, both in mammalian cells and *C. elegans*, although ECT2 and centralspindlin play essential roles in the completion of cytokinesis, they are dispensable for initiating furrowing (Raich et al., 1998; Tatsumoto et al., 1999; Jantsch-Plunger et al., 2000; Hirose et al., 2001; Matuliene and Kuriyama, 2002). In the present study, we demonstrated that centralspindlin and ECT2 induce the cleavage furrow through regulation of RhoA localization at the equatorial cortex, and centralspindlin is required for ECT2 localization at the cleavage furrow and central spindle microtubules. The molecular association of centralspindlin and ECT2 was also confirmed in mammalian cells. Taken together, our data suggest that the centralspindlin-ECT2-RhoA signaling pathway including their positional information is a common mechanism for the initiation of cytokinesis in animal cells.

The mechanism of centralspindlin localization at the tips of astral microtubules toward the equator requires elucidation. Since the motor activity of MKLP1 is required for cytokinesis (Matuliene and Kuriyama, 2002; Minestrini et al., 2003), centralspindlin might move toward the plus ends of astral microtubules and the motor activity at the tips may be suppressed to concentrate centralspindlin there. Since centralspindlin can persist only at the tips of equatorial astral microtubules, we speculate that turnover of astral microtubules toward the equator is slower as proposed (Canman et al., 2003) than that of other astral microtubules. At the tips of astral microtubules, however, ECT2 was not clearly detected. The mechanism of ECT2 recruitment to the cortex through centralspindlin remains elusive.

Moreover, in this model, RhoA would be expected to localize not only at the equatorial cortex but also at the central spindle microtubules because the same molecules are concentrated at both sites. RhoA was not observed, however, at the central spindle microtubules at any stage of cytokinesis (Fig. 1) and was not detected in immunoprecipitates of anti-centralspindlin or ECT2 antibodies (data not shown). We speculate that RhoA can interact with centralspindlin and ECT2 but only transiently. As RhoA activity is required for its localization at the equatorial cortex, active RhoA interacting molecules in the cortex appear to be responsible for stable RhoA localization at the cortex.

HsCYK4/MgcRacGAP has a GAP activity for Rho GTPases and suppresses Rac activity during cytokinesis, and this inhibition is essential for cytokinesis (Yoshizaki et al., 2003; D'Avino et al., 2004; Yoshizaki et al., 2004; D'Avino et al., 2005). However, HeLa cells overexpressing either dominant-negative or constitutively active forms of Rac1 divide normally in our experiments (data not shown). In addition, expression of a GAP-deficient mutant of HsCYK4 in U2OS cells caused inhibition of cytokinesis at the late stage (Lee et al., 2004), suggesting that GAP activity of HsCYK4 is required for completion of cytokinesis but not for initiation, at least in cultured cells.

Since another kinesin-like protein, MKLP2 is also known to be required for cytokinesis (Neef et al., 2003; Echard et al., 2004), we tested whether MKLP2 is involved in RhoA localization. Although depletion of MKLP2 causes cytokinesis failure at the late stage, RhoA localization at the equatorial cortex was not affected during the early stage of cytokinesis (data not shown). In addition, MKLP1/MKLP2 double knockdown did not enhance the effect on RhoA localization at the equatorial cortex by MKLP1 knockdown (data not shown), indicating that MKLP2 is not required for RhoA localization at cleavage plane determination.

In summary, our results identify a novel mechanism for RhoA localization during cytokinesis in which its activity and microtubule organization are required. We demonstrate a molecular mechanism that links microtubules with RhoA localization at the equatorial cortex. Centralspindlin detected at the tips of astral microtubules and central spindle microtubules, regulates ECT2 localization, and ECT2 is required for RhoA localization at the equatorial cortex that promotes proper furrow induction. These results provide evidence that the 'cleavage signals' from the mitotic apparatus include centralspindlin, ECT2 and RhoA.

Materials and Methods

Cell culture

Xenopus kidney epithelial A6 cells expressing *Xenopus* β-tubulin-GFP were provided by Y. Mimori-Kiyosue (Mimori-Kiyosue et al., 2000). They were grown at 23°C without CO_2 in 50% L-15 medium (GIBCO BRL). HeLa cells and NRK-52E cells obtained from Health Science Research Resources Bank (Oosaka, Japan) were cultured in DMEM (Dulbecco's modified Eagle's medium) (Sigma-Aldrich) at 37°C with 5% CO_2. All media were supplemented with 10% FBS (JRH BIOSCIENCE). These cells were cultured on coverslips (Matsunami) for immunofluorescence or in glass-bottom dishes (IWAKI) for live-cell imaging.

Antibodies

Primary antibodies used include mouse anti-RhoA (26C4), rabbit anti-MKLP1, goat anti-PRC1, rabbit anti-ECT2 (Santa Cruz Biotechnology), goat anti-MgcRacGAP (Abcam), mouse anti-α-tubulin (DM1A: Sigma-Aldrich), mouse anti-phosphorylated (Ser19) myosin light chain (Sakurada et al., 1994) provided by M. Seto (Asahi Kasei Corporation, Shizuoka, Japan), rabbit anti-HsCYK4 (Mishima et al., 2002) provided by M. Glotzer (University of Chicago, IL) and sheep anti-MKLP2 (Neef et al., 2003) provided by F. A. Barr (Max-Planck Institute, Martinsried, Germany). Secondary antibodies used include donkey anti-mouse, -rabbit or -goat antibodies conjugated with FITC, Cy3 or Cy5 (Jackson ImmunoResearch Laboratories). DAPI and Alexa Fluor 488 phalloidin (Molecular Probes) were used for DNA staining and actin filament staining, respectively. When necessary, FITC-conjugated anti-α-tubulin antibody (Sigma-Aldrich) or Rhodamine-conjugated anti-RhoA antibody (Santa Cruz Biotechnology) was used.

Immunofluorescence microscopy

For RhoA, MKLP1 and HsCYK4, cells were fixed with 10% TCA on ice for 15 minutes (Hayashi et al., 1999; Kosako et al., 2002; Yonemura et al., 2004). For PRC1, cells were fixed with methanol at –20°C for 30 minutes. For ECT2, cells were fixed with 4% formaldehyde in 10 mM HEPES (pH 7.4) at room temperature for 30 minutes. They were rinsed twice with phosphate-buffered saline containing 30 mM glycine (G-PBS), then treated with 0.2% Triton X-100 in G-PBS for 5 minutes to permeabilize cells. Then they were stained with antibodies as previously described (Yonemura et al., 2004). When cells were stained with two types of mouse monoclonal antibodies, one antibody and a secondary antibody were first used conventionally. After blocking with 1 mg/ml normal mouse serum for 5 minutes, cells were incubated with another mouse monoclonal antibody conjugated with FITC or Rhodamine. Images were acquired with an Olympus IX71 microscope or a Nikon E600 microscope equipped with an ORCA-ER camera (Hamamatsu Photonics) controlled by IPLab software (Scanalytics), using a UPlanApo 100×/1.35 NA oil Iris Ph3, PlanApo 60×/1.40 NA oil Ph3 (Olympus) or PlanApo 60×/1.40 NA oil Ph3 (Nikon) microscope. To measure RhoA fluorescence intensity, IPLab software was used. A Delta Vision (Applied Precision) deconvolution microscopy system equipped with an Olympus IX71 microscope and a cooled CCD camera (Series300 CH350; Photometrics) was used for deconvolution of images from serial optical sections. A total of 50-100 planes (0.2 μm slice) were captured for each cell, deconvolved and projected on a single plane by the SoftWorx system. Images were prepared for publication using Photoshop (Adobe).

Microinjection

C3 exoenzyme and GST-RhoGDI for microinjection experiments were produced and purified using pGEX-C3 provided by A. Hall (MRC Laboratory, London, UK)

and pGEX-2T carrying bovine RhoGDI cDNA provided by Y. Takai (Oosaka University, Oosaka, Japan). As a marker for microinjected cells, dextran fluorescein, 70,000 M_r, anionic, lysine fixable or dextran Rhodamine (Molecular Probes) was used. C3 (20 μg/ml) or GST-RhoGDI (9 mg/ml) with 1% fluorescently labeled dextran was microinjected into cells at prometaphase according to a method described previously (Yonemura et al., 2002). After a 30 minute incubation at 37°C, cells were fixed and stained for fluorescence microscopy.

RNA interference

Depletion of PRC1, HsCYK4, ECT2 or MKLP1 was performed using siRNA. The targeted sequence for human PRC1 cDNA (AF044588) was described previously (Mollinari et al., 2002) and synthesized using a Silencer™ siRNA Construction Kit (Ambion). The targeted sequence for HsCYK4 cDNA (BC032754) was 5′-GGGACTCTTCTTTGGTGAAGACTTT-3′; for human ECT2 cDNA (AY376439), 5′-TATCACTCTTGTTTCAATCTGAGGC-3′; for MKLP1 cDNA (X67155), 5′-AAAGAGTTCCTTCTGGGTGGTGTGA-3′; and for MKLP2 cDNA (BC012999), 5′-TTAGGTTGAAGAAGGATGCCTGTCC-3′ (synthesized by Invitrogen). HeLa cells plated on coverslips in 24-well plates or 3.5 cm dishes were transfected using OligofectAMINE™ (Invitrogen). To check the depletion of target proteins, cell lysates were analyzed by immunoblotting for anti-ECT2 (1:1000), HsCYK4 (1:1000), MKLP1 (1:1000) and GAPDH (1:5000; Abcam) antibodies or cells were fixed after 24-48 hours in culture at different time points and stained for immunofluorescence. RhoA images were recorded for cells where the target protein disappeared.

Drug treatment

For disruption of total microtubules, A6 cells expressing β-tubulin-GFP were treated with 16 μM nocodazole just after the onset of anaphase. For astral microtubule disruption, NRK-52E cells were treated with 150 nM nocodazole as reported (O'Connell and Wang, 2000). After a 3 hour incubation at 37°C in a CO_2 incubator, cells were fixed for immunofluorescence. Cells with mostly disrupted astral microtubules were selected for localization of RhoA. For disruption of actin filaments, HeLa cells were treated with 200 μM latrunculin A (Calbiochem). For inhibition of myosin II ATPase activity, HeLa cells were treated with 100 μM blebbistatin (Calbiochem).

Immunoprecipitation

To obtain cell fractions enriched in mitotic cells by synchronization, HeLa cells were treated with 5 mM thymidine (Sigma) for 12 hours, washed with PBS twice, incubated with fresh medium for 8 hours, treated with 5 mM thymidine for 12 hours again, and then incubated with fresh medium. After 9-10 hours, mitotic cells were mechanically shaken off from culture flasks and collected, and then incubated with fresh medium until cells entered anaphase. Cells were lysed in buffer A (20 mM HEPES, 150 mM NaCl, 1 mM $MgCl_2$, 5 mM EDTA, 1 mM DTT, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml cymostatin) with 0.1% Triton X-100 and the lysate was subjected to immunoprecipitation analysis as described (Mishima et al., 2002) except that protein G-Sepharose (Pharmacia) was used for anti-MKLP1 and anti-HsCYK4 antibodies.

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Note added in proof

While this manuscript was under review, four groups have also described results closely related to ours using sea urchin embryos (Bement et al., 2005) and cultured cells (Yüce et al., 2005; Zhao and Fang, 2005; Kamijyo et al., 2005).

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