

RhoA is required for cortical retraction and rigidity during mitotic cell rounding

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Mitotic cell rounding is the process of cell shape change in which a flat interphase cell becomes spherical at the onset of mitosis. Rearrangement of the actin cytoskeleton, de-adhesion, and an increase in cortical rigidity accompany mitotic cell rounding. The molecular mechanisms that contribute to this process have not been defined. We show that RhoA is required for cortical retraction but not de-adhesion during mitotic cell rounding. The mitotic increase in cortical rigidity also requires RhoA, suggesting that increases in cortical rigidity and cortical retraction are linked processes. Rho-kinase is also required

for mitotic cortical retraction and rigidity, indicating that the effects of RhoA on cell rounding are mediated through this effector. Consistent with a role for RhoA during mitotic entry, RhoA activity is elevated in rounded, preanaphase mitotic cells. The activity of the RhoA inhibitor p190RhoGAP is decreased due to its serine/threonine phosphorylation at this time. Cumulatively, these results suggest that the mitotic increase in RhoA activity leads to rearrangements of the cortical actin cytoskeleton that promote cortical rigidity, resulting in mitotic cell rounding.

Introduction

Mitotic cell rounding is the process in which a flat interphase cell changes shape to become more spherical in mitosis. This process occurs concurrent with nuclear envelope breakdown at the onset of mitosis and is completed before anaphase, when cells often elongate to a more oval shape before undergoing cytokinesis (for review see Glotzer, 2001). Mitotic cell rounding is likely important for chromosome segregation and the positioning of the cytokinetic furrow. Although it has been described for several types of cultured mammalian cells (Harris, 1973; Sanger et al., 1984; Cramer and Mitchison, 1997), a mechanism for mitotic cell rounding has not been well defined. Interestingly, it has been shown for several species that eggs become “more round” in mitosis. The mitotic surface contraction waves of *Xenopus laevis* eggs cause them to become taller and more spherical (Hara et al., 1980). Cortical rigidity measured with a suction pipet, and resistance to external pressure, increases as sea urchin eggs enter mitosis (Mitchison and Swann, 1955; Yoneda and Dan, 1972). Matzke et al. (2001) used atomic force microscopy to show that mammalian tissue culture cells (Ptk2) are more rigid in metaphase of mitosis than in interphase.

Mitotic cell rounding is accompanied by changes in the actin cytoskeleton. In interphase of many types of cultured cells, actin is predominantly organized into stress fibers that span the cytoplasm. Upon entry into mitosis, stress fibers disassemble and actin localizes primarily to the increasingly round cortex. Cramer and Mitchison (1997) showed that filamentous actin (F-actin) is required for coordinated retraction of the cell margin at the onset of mitosis, demonstrating that the actin cytoskeleton plays an active role in mitotic cell rounding. The enrichment of F-actin in the spherical cortex in mitosis could be favored by the cross-linking of actin filaments into a meshwork. Several actin-binding proteins can support such cross-linking, including filamin, spectrin, and α -actinin. Evidence that actin cross-linking promotes a rounded morphology comes from Cortese et al. (1989). The inclusion of filamin in actin-containing vesicles caused the vesicles to become smooth and spherical upon actin polymerization, whereas an irregular, angular morphology occurred in the absence of filamin (Cortese et al., 1989). Adhesions to the substrate are also altered in mitosis but remain connected to the cell via retraction fibers, which are exposed as the cell rounds. Structural and signaling proteins resident to focal adhesions become diffusely localized within the cytoplasm (Sanger et al., 1987; Hock et al., 1989; Yamakita et al., 1999). Plating cells on flexible substrates revealed that intracellular tension transmitted to the substrate through focal adhesions decreases during entry

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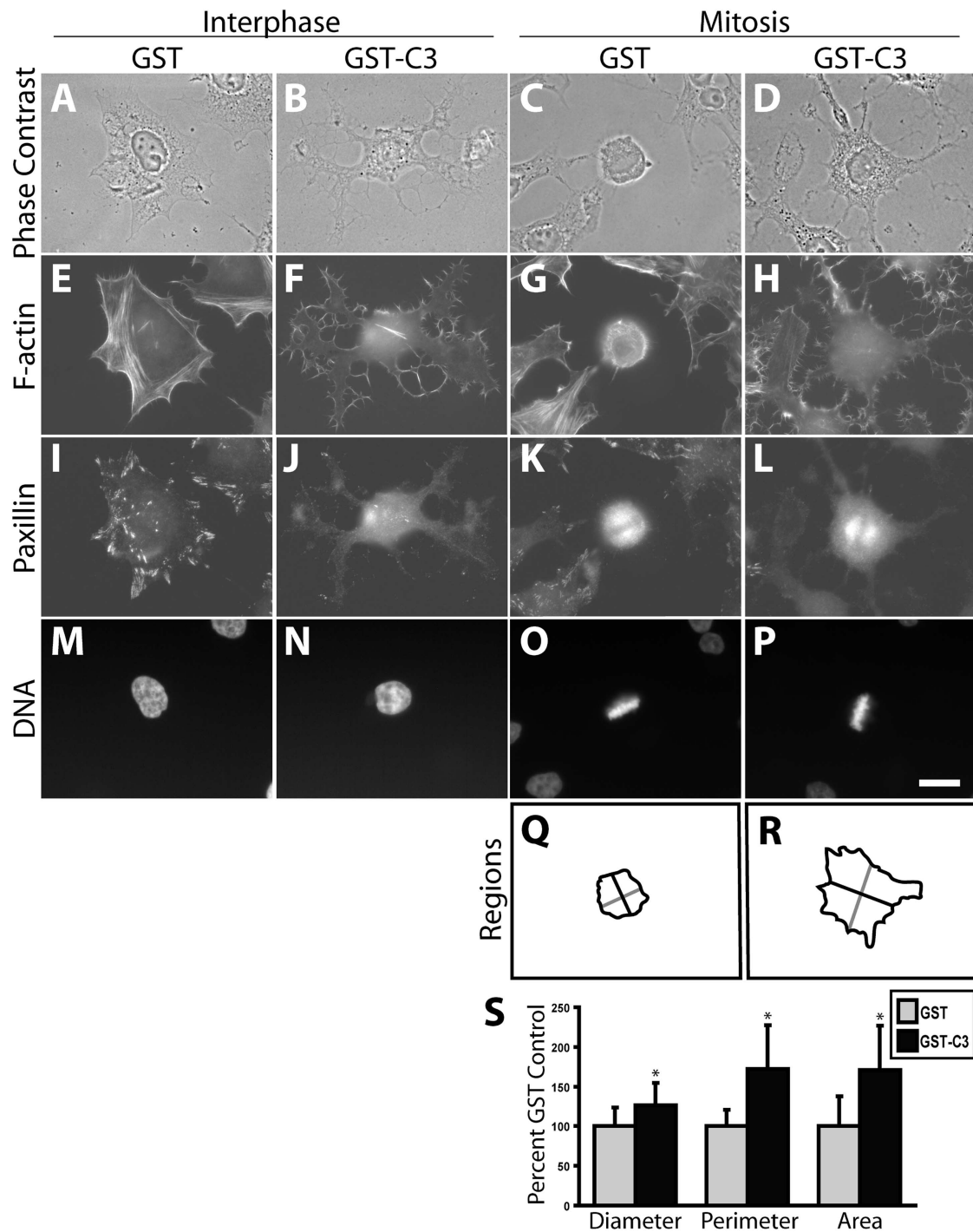


Figure 1. RhoA is required for cortical retraction but not de-adhesion during mitotic cell rounding. HeLa cells growing on glass coverslips were treated with recombinant GST-C3 toxin or GST alone (0.28 μg per 24-well plate well) by introduction with LipofectAmine reagent for 90 min. Cells were fixed and stained for F-actin, paxillin, and DNA. Cells in metaphase of mitosis were identified as having a metaphase plate of aligned chromosomes. For metaphase cells, diameter, perimeter, and area were measured by drawing on the phase image (C, D, Q, and R). The diameter measurement reported is the cell diameter (black line) bisecting and perpendicular to the diameter that passes through the metaphase plate (gray line). GST-treated metaphase cells are well rounded, whereas GST-C3-treated metaphase cells are not completely rounded (compare C with D). Bar, 15 μm . (S) Quantitation of measurements of mitotic cells. Measurements are averages and standard deviations from one of three equivalent experiments. $n \geq 21$ for each experiment. *, significant difference from GST ($P < 0.0005$).

into mitosis (Burton and Taylor, 1997). Here, we will refer to this disassembly of focal adhesions as de-adhesion.

The Rho family of small GTPases regulates actin organization and therefore cell shape (Van Aelst and D'Souza-

Schorey, 1997; Hall, 1998). One of the best-characterized members of this family is RhoA. Many RhoA effectors lead to remodeling of the actin cytoskeleton. The RhoA effector Rho-kinase stimulates the myosin II regulatory light chain

(MLC)* directly by phosphorylation and indirectly by inhibition of myosin phosphatase (Amano et al., 1996; Kimura et al., 1996). Another RhoA effector, citron kinase, also activates MLC by phosphorylation (Matsumura et al., 2001). Activation of MLC leads to actomyosin contractility, bundling, and cross-linking of actin filaments, and thus the formation and maintenance of actin stress fibers (Chrzanowska-Wodnicka and Burridge, 1996). The RhoA effector mDia, which promotes actin filament bundling, also contributes to proper stress fiber formation (Watanabe et al., 1997, 1999). Additionally, RhoA activity regulates the actin cytoskeleton by affecting actin filament assembly dynamics. RhoA, via Rho-kinase, stimulates LIM-kinase (LIMK), which down-regulates the actin-severing protein cofilin by phosphorylation (Maekawa et al., 1999; Sumi et al., 1999).

Inhibition of RhoA by treatment with C3 toxin causes dissolution of stress fibers and cell rounding in interphase cells (Paterson et al., 1990; Wieggers et al., 1991). The latter is thought to occur because inhibition of RhoA results in decreased focal adhesions and substrate adhesions in general. When RhoA is inhibited with C3 in mitotic cells, the actomyosin cytokinetic furrow is blocked (Kishi et al., 1993). Likewise, Y-27632, a specific inhibitor of Rho-kinase, causes dissolution of stress fibers and retraction of the cell margin (Uehata et al., 1997), and blocks MLC phosphorylation and furrow ingression during cytokinesis (Kosako et al., 2000). Interestingly, in earlier stages of mitosis, C3 treatment resulted in the spreading of the treated prophase cell as it was pulled by neighboring cells in a confluent monolayer of epithelial cells (O'Connell et al., 1999). The authors suggest that RhoA regulates the "mechanical integrity and strength of the cortex" (O'Connell et al., 1999). We hypothesized that RhoA mediates mitotic reorganization of the actin cytoskeleton, and that this rearrangement promotes cortical rigidity in mitosis and mitotic cell rounding.

Here we examine the role of RhoA in mitotic cell rounding. We show that RhoA is required for cortical retraction, but not de-adhesion during rounding. RhoA is also required for increases in cortical rigidity as cells enter mitosis, suggesting that cortical retraction and increased cortical rigidity are linked processes. Consistent with a role during this cell cycle transition, RhoA activity is elevated in preanaphase mitotic cells compared with interphase cells. The negative regulator of RhoA, p190RhoGAP, is less active at metaphase than in interphase and this down-regulation may play a role in mitotic stimulation of RhoA.

Results

RhoA is required for cortical retraction but not de-adhesion during mitotic cell rounding

Mitotic cell rounding is comprised of at least two independent processes: de-adhesion and cortical retraction. De-adhesion is the dissolution of stress fibers and focal adhesions and the dispersal of the component proteins. This has been described for several stress fiber and focal adhesion proteins

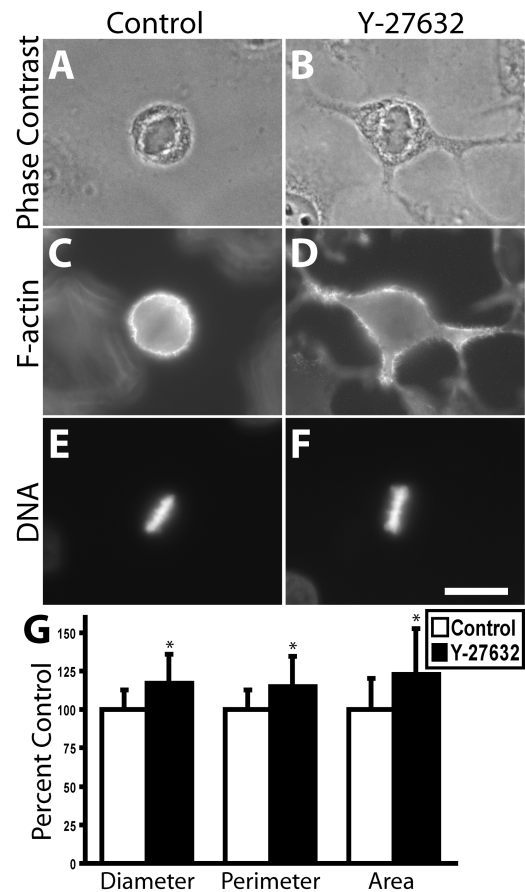


Figure 2. Rho-kinase is required for cortical retraction during mitotic cell rounding. HeLa cells were treated with Y-27632 or vehicle alone (Control) in growth medium for 1 h. Cells were fixed and stained for F-actin and DNA. Phase contrast and fluorescence images are shown. Bar, 15 μ m. (G) Cortical retraction was quantified as for Fig. 1. Measurements are averages and standard deviations from one of three equivalent experiments. $n \geq 30$ for each experiment. *, significant difference from Control ($P < 0.0005$).

(Sanger et al., 1987; Hock et al., 1989; Sanger et al., 1989). During cortical retraction, the cell cortex changes from well spread and flattened to spherical and refractile by phase contrast imaging. To examine whether RhoA has a role in either of these processes, we treated HeLa cells with a GST fusion with C3 toxin (C3), an inhibitor of RhoA. This treatment significantly reduces RhoA-GTP levels but does not inhibit the related GTPase Rac1 (unpublished data). To test for a role in de-adhesion, we first stained control and C3-treated cells with an antibody to paxillin, a component of focal adhesions, and with phalloidin to follow changes in the actin cytoskeleton. To ensure that cells were at or near the end-point of mitotic rounding, only cells with a metaphase plate of condensed, aligned chromosomes were imaged (Fig. 1, O and P). We found that in C3-treated cells and GST-treated control cells, paxillin is diffusely localized in mitotic cells (Fig. 1, compare I with K and J with L). Also, actin stress fibers are absent in mitotic C3-treated cells, as in control cells (Fig. 1, compare E with G and F with H). This suggests that RhoA activity is not required for de-adhesion.

The phase contrast and phalloidin staining images of mitotic cells treated with C3 show that cortical retraction fails

*Abbreviations used in this paper: GAP, GTPase activating protein; GEF, guanine nucleotide exchange factor; LIMK, LIM-kinase; MLC, myosin II regulatory light chain; PPI, protein phosphatase 1.

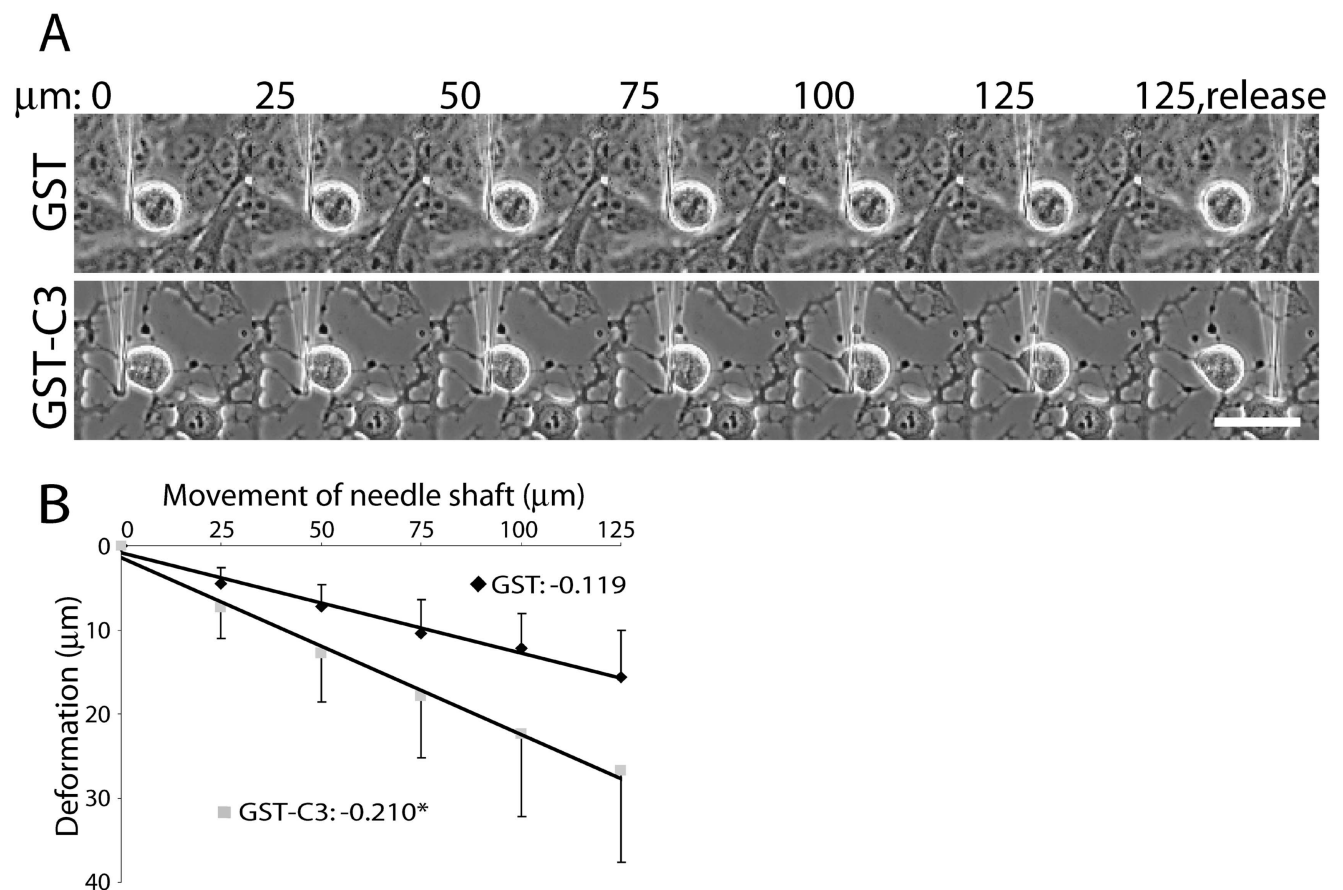


Figure 3. RhoA is required for rigidity of rounded mitotic cells. (A) Phase images of HeLa cells and the microneedle show the needle in contact with the cell before application of pressure (0) and after needle movement of various distances (shown in microns across the top). The location of the needle shaft after movement of 125 μm and release from the cell by lifting is shown in the last frame of each panel. A cell treated with GST (top panel) is not deformed by pressure exerted by the needle. A cell treated with GST-C3 (bottom panel) is deformed by pressure more than the GST-treated cell; the needle tip moves further when a given amount of pressure is applied to the GST-C3 cell than when it is applied to the GST-treated cell. Bar, 125 μm . (B) Cell rigidity is plotted as force versus distance of deformation (see Materials and methods). Data points are averages and standard deviations (GST, black diamonds; GST-C3, gray squares). Linear regressions are plotted; slopes correspond to cell rigidity. *, significant difference from GST treatment ($P < 0.0005$). $n > 25$ for each treatment. The values for Y-27632 treatment are included in the text.

to result in a spheroid cell shape in these RhoA-inhibited cells (Fig. 1, C, D, G, and H). Rather, mitotic C3-treated cells have undergone a similar degree of cortical retraction as interphase C3-treated cells (Fig. 1, B and D). To quantify the involvement of RhoA in cortical retraction, we compared the area, perimeter, and diameter of cells treated with C3 to that of control GST-treated cells (Fig. 1, Q and R; see legend for more measurement details). We found that by all three criteria, cortical retraction was significantly inhibited in C3-treated cells compared with cells treated with GST alone (Fig. 1 S; Diameter: GST, $26.6 \pm 6.3 \mu\text{m}$; GST-C3, $33.6 \pm 7.5 \mu\text{m}$; Perimeter: GST, $86.1 \pm 17.9 \mu\text{m}$; GST-C3, $148.0 \pm 47.7 \mu\text{m}$; Area: GST, $435.4 \pm 162.7 \mu\text{m}^2$; GST-C3, $744.4 \pm 241.8 \mu\text{m}^2$). This was also seen with CHO and A431 cells (unpublished data). In contrast to the decreased rounding seen with mitotic cells treated with C3, interphase cells treated with C3 were more rounded and refractile than GST-treated cells and had decreased actin stress fibers and focal adhesions, as has been observed previously (Paterson et al., 1990; Wieggers et al., 1991; Fig. 1, A, B, E,

F, I, and J). Phalloidin staining of F-actin also showed that C3-treated mitotic cells lack the circular band of F-actin seen in GST-treated rounded mitotic cells (Fig. 1, G and H). From these results, we conclude that RhoA is required for cortical retraction, but not de-adhesion, during mitotic cell rounding, and that the requirement for RhoA in cortical retraction may involve RhoA-regulated actin reorganization.

Inhibition of Rho-kinase also results in defects in cortical retraction during mitotic cell rounding

Rho-kinase is a RhoA effector that plays an important role in transducing signals from RhoA to the actin cytoskeleton (Leung et al., 1995; Ishizaki et al., 1996; Kimura et al., 1996). Therefore, we investigated whether Rho-kinase is also required for cortical retraction during mitotic cell rounding. HeLa cells were treated with the Rho-kinase inhibitor Y-27632, and cells in metaphase of mitosis were assessed for degree of rounding by measuring diameter, perimeter, and area. As was the case for the C3-treated cells, by all three criteria, cortical retraction was significantly inhibited

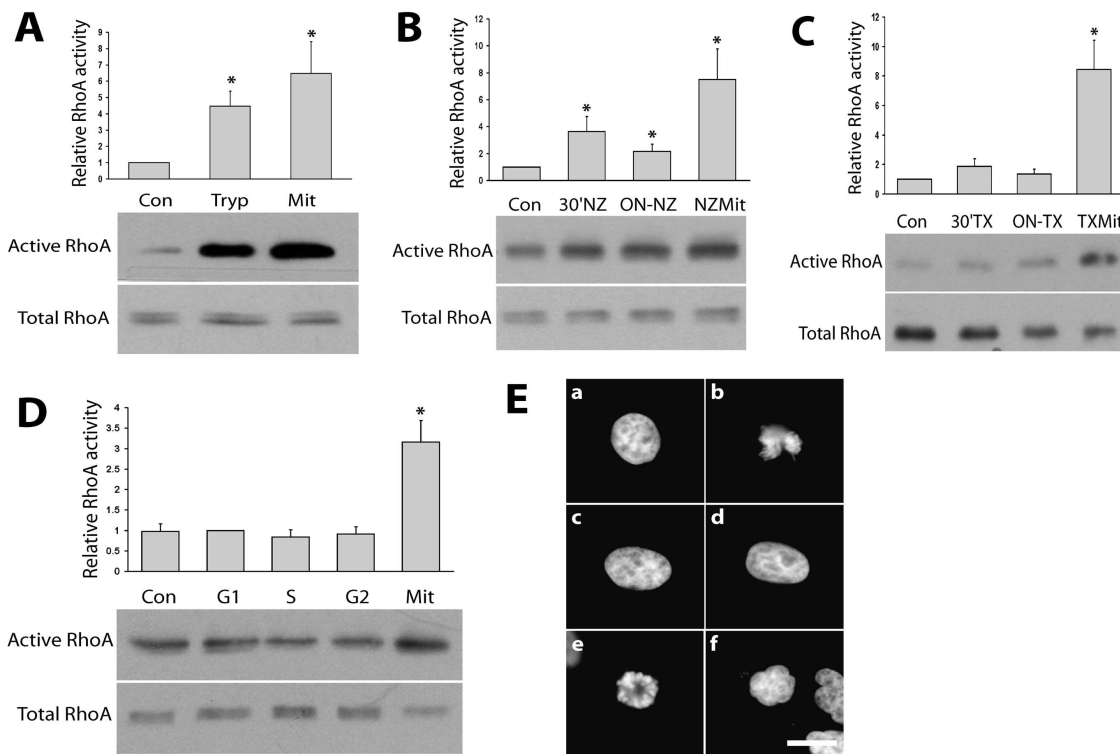


Figure 4. RhoA activity is elevated in mitosis. (A) RhoA activity was measured in lysates from control interphase adherent HeLa cells (Con), cells that had been rounded with trypsin (Tryp), or mitotic cells harvested by the knock-off method from untreated asynchronous cultures (Mit). RhoA immunoblots show RhoA protein levels in pull-downs (Active RhoA) and lysates (Total RhoA). *, significant difference from control (for all panels) ($P < 0.05$). Bars represent SEM. All data shown are representative of at least five replicate experiments. (B) HeLa cells were treated with nocodazole for 30 min (30'NZ) or overnight. For overnight treatment, rounded mitotic cells were harvested by knock-off (NZMit), with the remaining adherent cells in interphase (ON-NZ). (C) HeLa cells were treated with taxol for 30 min (30'TX) or overnight. Rounded mitotic cells (TXMit) that had accumulated during the overnight treatment were harvested by knock-off. Remaining interphase cells comprised the overnight interphase (ON-TX) sample. (D) HeLa cells were synchronized in G1 by a single thymidine block followed by a brief nocodazole block and then a washout to allow progression through mitosis and into G1 of interphase. Cells were synchronized in S phase with a double thymidine block, and in G2 with a double thymidine block followed by a 5-h washout. Rounded mitotic cells were harvested from cultures that were treated with taxol for 4 h after washout from a single thymidine block. (E) Staining of HeLa cell nuclei with Hoechst shows normal morphology with all treatments. (E, a) Untreated interphase cell, (b) untreated mitotic cell, (c–f) treated with 10 nM taxol, (c) 30' treatment (interphase cell), (d) overnight treatment (interphase cell), (e) overnight treatment (mitotic cell), (f) overnight treatment (cells harvested as rounded mitotic cells and fixed 7 h after washout, now interphase). Note that taxol does not cause the nuclear condensation characteristic of apoptosis at the concentration and time course used. Bar, 15 μm .

in Y-27632-treated cells compared with controls (Fig. 2 G; Diameter: Control, $17.0 \pm 2.2 \mu\text{m}$; Y-27632, $19.9 \pm 3.2 \mu\text{m}$; Perimeter: Control, $53.7 \pm 6.8 \mu\text{m}$; Y-27632, $61.9 \pm 10.6 \mu\text{m}$; Area: Control, $206.6 \pm 42.2 \mu\text{m}^2$; Y-27632, $254.8 \pm 60.7 \mu\text{m}^2$). Phalloidin staining of F-actin reveals that the intense circular band on actin in control mitotic cells is absent in Y-27632-treated cells (Fig. 2, C and D). These results show that Rho-kinase is likely a key effector that mediates the RhoA dependency of cortical retraction during mitotic cell rounding.

RhoA and Rho-kinase are required for cortical rigidity in rounded preanaphase mitotic cells

In addition to cell rounding, entry into mitosis is also accompanied by an increase in cell rigidity (Mitchison and Swann, 1955; Yoneda and Dan, 1972; Matzke et al., 2001). Because it seemed likely that the same reorganization of the cortical cytoskeleton is required for cortical retraction during cell rounding and mitotic cortical rigidity, we hypothesized that RhoA is required for both processes. To determine whether

RhoA is required for cells to become more rigid as they enter mitosis, we treated cells with C3, as for Fig. 1, and tested the rigidity of preanaphase mitotic cells (identified as having a metaphase plate of chromosomes by phase contrast imaging). To do this, a micromanipulation needle was brought into contact with the subject cell, and controlled increments of pressure were exerted on the cell by moving the needle shaft set distances. Images of the location of the needle tip and cell were captured and used to quantify the cells' resistance to pressure. Control mitotic cells resist pressure; they are not deformed by the needle (Fig. 3 A, top). Under the same amounts of pressure, a C3-treated mitotic cell is deformed visibly by the needle (Fig. 3 A, bottom). Cell rigidity is significantly decreased by the C3 treatment (Fig. 3 B; GST: -0.119 , GST-C3: -0.210 ; see Materials and methods for quantitation details). Mitotic cells treated with Y-27632 to inhibit Rho-kinase are significantly less rigid than control cells (Control: -0.237 ± 0.098 ; Y-27632: $-0.425 \pm 0.14^*$; an asterisk indicates a significant difference from control; $P < 0.0005$.) From these results, we conclude that RhoA and

Rho-kinase are required for both cortical retraction and increases in cortical rigidity as cells enter mitosis, and that these processes are likely linked.

RhoA activity is higher in preanaphase mitotic cells than in interphase cells

The fact that RhoA is required for both cortical retraction and increases in cortical rigidity as cells enter mitosis suggests that RhoA activity increases during this cell cycle transition. To test this, we compared the activity of RhoA in spread interphase cells with that in rounded mitotic cells. Rounded mitotic HeLa cells were harvested from untreated asynchronous cultures by mechanical disruption. The adherent cells remaining after this “knock-off” procedure were in interphase and comprised the control sample (Fig. 4 E, a and b). To determine the degree of RhoA activation in each condition, we isolated active, GTP-bound RhoA from cell lysates using an affinity matrix consisting of the RhoA-binding domain of the RhoA effector Rhotekin fused to GST and immobilized on sepharose beads (Ren et al., 1999). Active RhoA associated with the beads, and total RhoA in lysates was detected by immunoblotting. We found that, whereas the total level of RhoA did not change, there was significantly more active RhoA in lysates from mitotic cells than in lysates prepared from interphase controls (Fig. 4 A). The amount of active RhoA was also elevated in trypsin-rounded interphase cells, as has been seen (Ren et al., 1999).

The mitotic population harvested from untreated cultures contained cells at all stages of mitosis. It has been shown that RhoA activity increases during, and is required for, cytokinesis (O’Connell et al., 1999; Kimura et al., 2000). Therefore, it was important to separate preanaphase cells (at the endpoint of cell rounding) from post-anaphase cells (preparing for or performing cytokinesis). A common technique for accumulating cells in mitosis is to use microtubule-disrupting drugs, which prevent normal spindle assembly and progression from metaphase into anaphase. However, it has been shown that microtubule-depolymerizing drugs cause increases in RhoA-GTP activity (Ren et al., 1999; Fig. 4 B). Therefore, we used taxol treatment to accumulate mitotic cells. Taxol is a microtubule-stabilizing agent that also causes cells to accumulate in mitosis at preanaphase but does not stimulate RhoA activity (Ren et al., 1999; Fig. 4 C). Significantly, cells arrested in preanaphase mitosis with taxol had high RhoA activity (Fig. 4 C). This result was also seen with REF2A rat embryo fibroblasts (unpublished data). The cells remaining in interphase after longer taxol treatments accumulated intracellular taxol and did not have elevated RhoA activity (Jordan et al., 1993; Fig. 4 C). Therefore, we conclude that taxol treatment alone does not affect RhoA activity, and that RhoA activity is elevated in mitosis before anaphase and cytokinesis.

Hoechst staining of DNA shows that cells treated with this low level of taxol (10 nM) were not undergoing apoptosis (Fig. 4 E, c–f). Interphase cells treated with taxol for 8–12 h have normal nuclear morphology (Fig. 4 E, d). Mitotic taxol-treated cells had condensed chromatin before taxol washout (Fig. 4 E, e). At 7 h after taxol washout, nuclei have interphase morphology but are lobulated (Fig. 4 E, f). This may be due to the abnormal mitoses (multipolar spindles) that occur in taxol-treated cells (Brown et al., 1985).

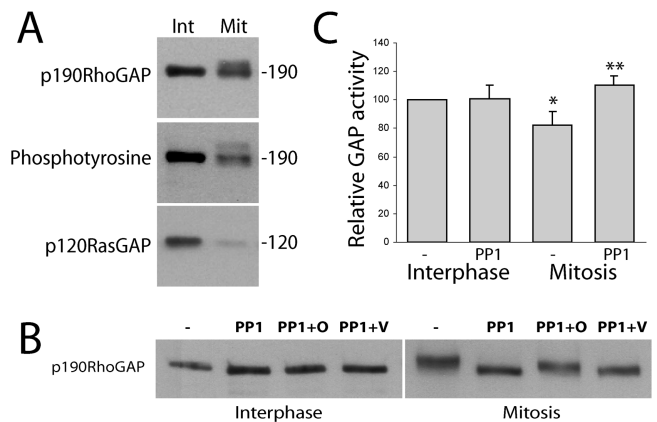


Figure 5. p190RhoGAP is tyrosine dephosphorylated, serine/threonine phosphorylated, and decreased in activity in mitosis. (A) p190RhoGAP was immunoprecipitated from HeLa cells and probed, stripped, and reprobbed for p190RhoGAP, phosphotyrosine, and coassociation with p120RasGAP. Note the decrease in phosphotyrosine and p120RasGAP associated with mitotic p190RhoGAP, and the electrophoretic mobility shift in mitosis. (B) p190RhoGAP was immunoprecipitated from interphase and mitotic HeLa cells, and immunoprecipitates were washed into PP1 reaction buffer and divided into four equal samples. One sample was not treated (–); the remaining three each received 1 U PP1 phosphatase. The serine/threonine phosphatase inhibitor okadaic acid (PP1 + O) and the tyrosine phosphatase inhibitor vanadate (PP1 + V) were added to one sample each before PP1 was added. Note that the retarded electrophoretic mobility of mitotic p190RhoGAP is restored to the interphase mobility by PP1 treatment. (C) p190RhoGAP activity is lower in mitosis than in interphase as assayed for RhoA GAP activity. PP1 phosphatase treatment increases the activity of mitotic p190RhoGAP. Graphed are means from six experiments. Bars represent the SEM. *, significant difference from Interphase (–); **, significant difference from Mitosis (–) ($P < 0.05$).

We next examined whether RhoA activity is elevated at any other stage of the cell cycle besides the elevation that occurs during mitosis. We assayed RhoA activity in HeLa cells synchronized in G1, S phase, and G2 and compared RhoA activity at these times with that in mitosis and in asynchronous cells. There is no significant difference in RhoA activity among the various cell cycle phases other than in mitosis (Fig. 4 D). From these results, we conclude that RhoA activity increases as cells enter mitosis. Combined with our previous data, these results further suggest that increased RhoA activity leads to increased cortical rigidity and cortical retraction, which cause cell rounding at the onset of mitosis.

p190RhoGAP activity is lower in mitosis than in interphase

Rho proteins are regulated by two major families of proteins: guanine nucleotide exchange factors (GEFs), which activate Rho proteins by loading them with GTP, and GTPase activating proteins (GAPs), which inactivate Rho proteins by promoting their intrinsic GTP hydrolysis activity (for review see Bishop and Hall, 2000). Also, guanine nucleotide dissociation inhibitors interact with and inhibit Rho GTPases (Bishop and Hall, 2000). The mitotic increase in RhoA activity is most likely due either to the activation of a GEF or the inactivation of a GAP (Vincent and Settleman, 1999). The recent observation that the decrease in RhoA activity

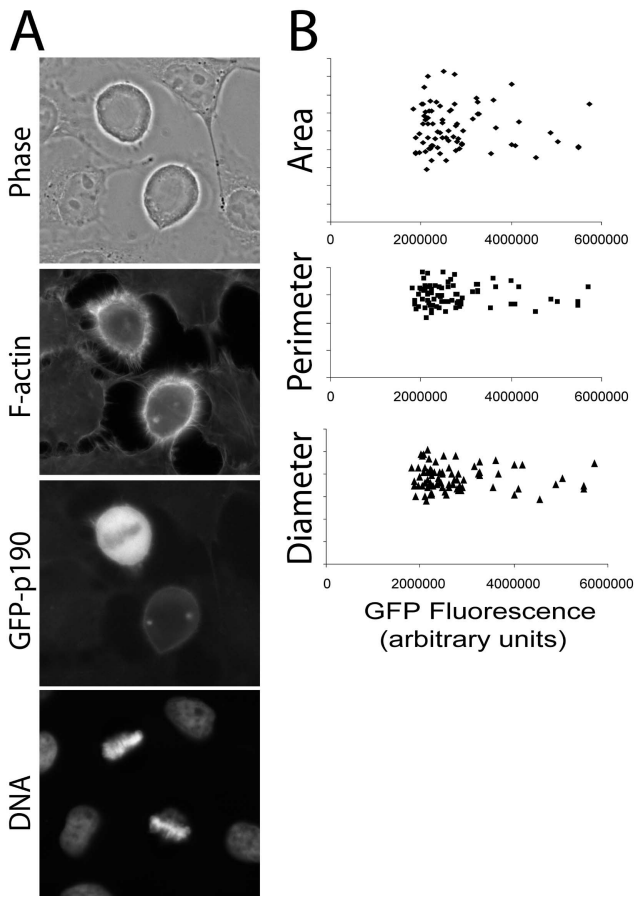


Figure 6. **Transient overexpression of GFP-p190RhoGAP does not block mitotic cell rounding.** At 24 h after transfection, HeLa cells were fixed, stained, imaged, and measured as for Figs. 1 and 2. Also measured was the total GFP fluorescence within a circular region of fixed size, which comprised much of the cytoplasm. (A) A metaphase cell expressing GFP-p190RhoGAP has undergone mitotic cell rounding to a similar extent as a neighboring cell expressing little or no GFP-p190RhoGAP. (B) The extent of mitotic cell rounding, measured as cell diameter, perimeter, and area, was plotted against the intensity of the cytoplasmic GFP signal. GFP fluorescence is not predictive of the three cell rounding measurements.

after integrin engagement is mediated by p190RhoGAP (Arthur et al., 2000) led us to investigate whether this negative regulatory protein was also responsible for changes in RhoA activity in mitosis, another incidence of adhesion dynamics.

Tyrosine phosphorylation of p190RhoGAP has been correlated with its activity (Fincham et al., 1999; Arthur et al., 2000; Dumenil et al., 2000). Immunoprecipitated p190RhoGAP bears less tyrosine phosphorylation in mitosis than in interphase (Fig. 5 A). Also, decreased p120RasGAP is present in p190RhoGAP immunoprecipitates in mitosis (Fig. 5 A). These findings suggest that mitotic p190RhoGAP has decreased activity (Trouiliaris et al., 1995). Careful inspection of immunoprecipitated p190RhoGAP revealed that it migrates more slowly and as a more diffuse band in mitosis (Fig. 5, A and B). Changes in gel migration often reflect altered levels of serine/threonine phosphorylation. Therefore, immunoprecipitates were treated with protein phosphatase 1 (PP1). This treatment restored the mobility

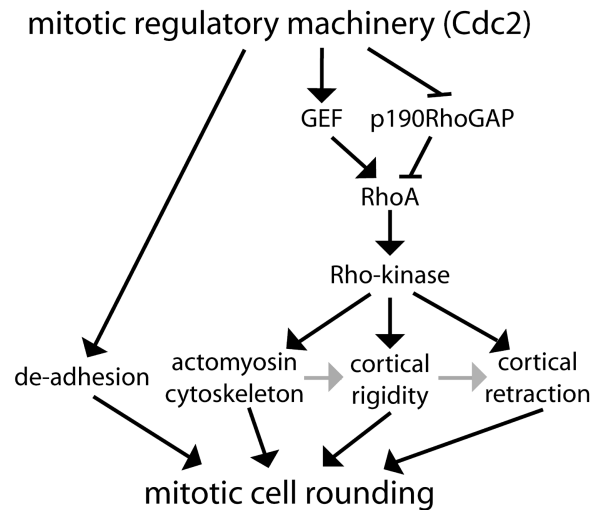


Figure 7. **RhoA and Rho-kinase are required for actin reorganization, cortical rigidity, and cortical retraction during mitotic cell rounding.** Black arrows denote direct or indirect stimulation as shown here or in the literature. Gray arrows represent possible causal relationships. Black lines followed by bars denote inhibition.

of mitotic p190RhoGAP to that of p190RhoGAP from interphase cells (Fig. 5 B). These results suggest that in mitosis, p190RhoGAP has decreased phosphotyrosine, decreased association with p120RasGAP, and an altered electrophoretic mobility due to increased phosphoserine or phosphothreonine.

Because the activation state of p190RhoGAP that is tyrosine dephosphorylated and serine/threonine phosphorylated has not been characterized, it was necessary to directly measure the GAP activity of p190RhoGAP isolated from mitotic cells. For these experiments, recombinant RhoA loaded with [γ - 32 P]GTP was incubated with immunoprecipitated p190RhoGAP. Mitotic p190RhoGAP was found to be less active than p190RhoGAP isolated from interphase cells (Fig. 5 C). When mitotic p190RhoGAP was incubated with PP1 phosphatase, its RhoA GAP activity was increased over that of untreated mitotic p190RhoGAP, and to the activity level of interphase p190RhoGAP (Fig. 5 C). These data demonstrate that mitotic phosphorylation of p190RhoGAP is required for the down-regulation of p190RhoGAP that occurs in mitosis. In addition, the mitotic decrease in phosphotyrosine and association with p120RasGAP and the phosphoserine/threonine-mediated alteration in electrophoretic mobility of p190RhoGAP correlate with decreased GAP activity of p190RhoGAP.

The results presented above suggest that mitotic down-regulation of p190RhoGAP plays a role in RhoA-dependent mitotic cell rounding. To test whether this is true, we attempted to override mitotic down-regulation of p190RhoGAP. GFP-tagged wild-type p190RhoGAP was transiently overexpressed in HeLa cells and mitotic cell rounding was measured using images of cells in metaphase of mitosis. Imaging demonstrated that cells expressing GFP-p190RhoGAP undergo comparable mitotic cell rounding to nonexpressors (Fig. 6 A). Quantitation also revealed that the level of expression of p190RhoGAP was not predictive of cell size at the endpoint of mitotic cell rounding (Fig. 6 B). There-

fore, using this method, we were unable to show that mitotic down-regulation of p190RhoGAP contributes to mitotic cell rounding.

Discussion

RhoA and Rho-kinase are required for cortical retraction and rigidity in mitotic cell rounding

In this paper, we show that RhoA is required for cortical retraction and rigidity as cells enter mitosis (results summarized in Fig. 7). The idea that RhoA regulates cortical rigidity is supported by several published observations. Treatment of interphase cells with C3 toxin causes a collapse of the spread morphology and the exposure of thin retraction fibers that branch and bleb (Rubin et al., 1988; Chardin et al., 1989). Our observation that RhoA is required for cortical rigidity during mitosis is also consistent with the finding that epithelial cells injected with C3 in prophase of mitosis appear to increase greatly in area as, presumably, they are stretched by neighboring cells (O'Connell et al., 1999).

The mechanism of RhoA activation in mitosis

We found that RhoA activity is elevated in preanaphase mitotic cells. Our results differ from those of Kimura et al. (2000), who reported that the activity of RhoA was the same in nocodazole-arrested mitotic cells as that in S phase of interphase. Currently, we cannot explain the apparent discrepancy. However, the results of this previous study are surprising because they did not observe the increase in RhoA activity induced by microtubule depolymerization as described by us in this work and by other studies (Enomoto, 1996; Zhang et al., 1997; Liu et al., 1998; Ren et al., 1999; Krendel et al., 2002). The difference may be due to Kimura et al. (2000) using the active RhoA-binding domain of mDia, rather than Rhotekin, as was used here. We found that p190RhoGAP activity is lower in mitosis than in interphase, which correlates with the mitotic increase in the activity of RhoA. Also, serine/threonine phosphorylation of p190RhoGAP is elevated in mitosis, and this phosphorylation is required for mitotic down-regulation of p190RhoGAP. Tyrosine phosphorylation of p190RhoGAP is reduced in mitosis. Src, which stimulates p190RhoGAP activity by phosphorylation on tyrosines (Fincham et al., 1999), has been shown to be highly active in mitosis (Chackalaparampil and Shalloway, 1988). It is possible that mitotic serine/threonine phosphorylation of p190RhoGAP, or elevated protein tyrosine phosphatase activity in mitosis, blocks stimulatory phosphorylation on tyrosine residues.

The results presented in Fig. 5 suggested that this regulation of p190RhoGAP plays a role in RhoA-dependent mitotic cell rounding. However, transient expression of p190RhoGAP did not block rounding (Fig. 6). Importantly, the mitotic cells that were measured likely expressed only low levels of p190RhoGAP and consequently may still have had adequate levels of active RhoA. Cells expressing high levels of GFP-p190RhoGAP were highly retracted in interphase or were apoptotic, and it is doubtful that many of these cells entered mitosis. Also, it is possible that the exogenous p190RhoGAP was properly down-regulated, and that RhoA was activated sufficiently to elicit mitotic round-

ing. Therefore, this approach cannot conclusively address whether p190RhoGAP down-regulation in mitosis is required for mitotic cell rounding. We also found that mitotic cell rounding was not blocked in Rat1 cells stably overexpressing p190RhoGAP at three to five times the endogenous levels (unpublished data). However, treatment with C3 revealed that mitotic cell rounding is RhoA independent in these cells. This indicates that, at least in Rat1 cells, there is an additional Rho-independent mechanism for this process. Interestingly, both the elevation of p190RhoGAP and C3 treatment increased blebbing in mitotic Rat1 cells, consistent with a role for RhoA in regulating cortical rigidity during mitosis in these cells. Therefore, we conclude that mitotic activation of RhoA may be due in part to p190RhoGAP down-regulation, but that a Rho GEF likely contributes to mitotic RhoA activation and cell rounding (Fig. 7).

How does RhoA activation promote mitotic cell rounding?

Studies demonstrating mitotic cortical rigidity (Mitchison and Swann, 1955; Yoneda and Dan, 1972; Matzke et al., 2001) have not addressed its cause. Among the factors that could affect this physical characteristic are cytoskeletal contractility under the cortex, actin filament cross-linking, and the degree of coupling between the cortical cytoskeleton and the membrane. RhoA, a major regulator of the actin cytoskeleton, can affect all three of these characteristics. We propose that RhoA and Rho-kinase promote mitotic cortical retraction and rigidity through regulation of the actin cytoskeleton (Fig. 7). RhoA regulates the actin cytoskeleton in many ways. Most attention has been directed toward the RhoA-dependent Rho-kinase stimulation of phosphorylation of MLC and the consequent stimulation of myosin contractility (Amano et al., 1996; Kimura et al., 1996). It was recently shown that the rounded shape of FAK^{-/-} cells is alleviated by inhibition of Rho-kinase by Y-27632, another example where a rounded morphology is dependent on RhoA and Rho-kinase activity (Chen et al., 2002). Yamakita et al. (1994) showed that in preanaphase mitotic cells, the majority of MLC is phosphorylated (possibly by Cdc2 [Satterwhite et al., 1992] or a conventional PKC [Varlamova et al., 2001]) on residues that confer inhibition. Inhibitory phosphorylation of MLC blocks phosphorylation on stimulatory residues (Nishikawa et al., 1984). Therefore, even in the presence of high RhoA activity in mitosis, inhibitory phosphorylation would block stimulatory phosphorylation by Rho-kinase. Interestingly, Yamakita et al. (1994) report that although 31% of MLC is phosphorylated early in mitosis, only 15% of MLC is phosphorylated on inhibitory residues at this time. This would seem to allow for the possibility that some MLC in mitosis bears stimulatory phosphorylation and is actively contractile. In addition, Shuster and Burgess (1999) showed that at no time does cortical MLC bear inhibitory phosphorylation, and that in metaphase of mitosis, some cortical MLC bears stimulatory phosphorylation. Further evidence for the presence of myosin contractility in preanaphase mitosis comes from Sanger et al. (1989), who performed time-lapse microscopy of mitosis in cells that had been microinjected with fluorescently labeled MLC. The labeled myosin incorporated into and lo-

calized to puncta along stress fibers. As cells entered mitosis and underwent mitotic cell rounding, stress fibers shortened and individual myosin-containing puncta along stress fibers became closer together. Microinjected fluorescent α -actinin had a similar behavior (Sanger et al., 1987). This observation of actomyosin contractility at this stage of the cell cycle demonstrates that myosin contractility could contribute to the state of the cortical actin cytoskeleton at this time.

Several other Rho-kinase substrates also regulate the actin cytoskeleton. Adducin, which links F-actin to the plasma membrane through spectrin, is stimulated by phosphorylation by Rho-kinase (Kimura et al., 1998; Fukata et al., 1999). The resulting enhancement of coupling between the actin cytoskeleton and the spectrin-based membrane cytoskeleton could promote mitotic cortical rigidity and rounding. Cofilin, an actin-severing protein that promotes depolymerization of F-actin, is down-regulated by RhoA signaling through Rho-kinase and inhibitory phosphorylation by LIMK (Maekawa et al., 1999). It has recently been shown that cofilin activity in mitosis is lower than in interphase, but then decreases further before increasing to interphase levels as cells undergo cytokinesis (Amano et al., 2002). Part of this multiphasic regulation has been shown to be independent of Rho-kinase and LIMK (Amano et al., 2002). However, the possibility exists that elevated RhoA activity contributes to the down-regulation of cofilin in mitosis. This would lead to a stabilization of the actin cytoskeleton and rigidity in rounded mitotic cells.

The formation and maintenance of stress fibers and focal adhesions are stimulated by and require RhoA activity (Chardin et al., 1989; Ridley and Hall, 1992). However, in mitosis, stress fibers and the associated focal adhesions disassemble in the presence of high RhoA activity. Therefore, the disassembly of these structures must be driven by a mitotic pathway independent of RhoA activity. One candidate mechanism is down-regulation of myosin contractility, which promotes these structures. However, as discussed above, the state of myosin activity during mitotic cell rounding has not been clearly defined. Another possibility is that an actin-binding protein disrupts the interaction of actin with myosin. Perhaps mitotic regulation of caldesmon causes stress fiber disassembly in the presence of high RhoA activity (Yamashiro et al., 1991). Because actomyosin contractility is required to maintain focal adhesions, stress fiber disassembly alone may be sufficient for focal adhesion disassembly in mitosis. However, mitotic phosphorylation of the cytoplasmic focal adhesion proteins FAK, p130cas, and paxillin could contribute to focal adhesion disassembly (Yamaguchi et al., 1997; Yamakita et al., 1999). Alternatively, it is possible that there are spatially distinct pools of RhoA activity in the cell during mitotic cell rounding. We are currently investigating the mechanism for the mitotic disassembly of stress fibers and focal adhesions in the presence of high overall RhoA activity.

It has long been known that most animal cells round up during mitosis. Why is mitotic cell rounding such a universal phenomenon? Although there is no evidence for a "cell rounding checkpoint," Rieder et al. (1994) observed that the height of PtK1 epithelial cells (i.e., their degree of rounding) correlated inversely with the time taken to progress from nu-

clear envelope breakdown to anaphase onset. This suggests that cell rounding facilitates the assembly and function of the mitotic spindle. Mitotic cell rounding might prevent aneuploidy and delays in mitosis by helping to center the spindle, bringing the spindle poles together, and preventing chromosome mono-orientation. We propose that in mitosis, RhoA, via Rho-kinase, causes the cortex to become rigid and spherical in shape, and thereby promotes the fidelity and speed of chromosome alignment and segregation.

Materials and methods

Cell culture and synchronization

HeLa cells were maintained in DME with 10% fetal bovine serum, supplemented with 10 U/ml penicillin, 10 μ g/ml streptomycin, 0.25 μ g/ml amphotericin B as fungizone. Nocodazole and taxol (Paclitaxel) (Sigma-Aldrich) were diluted in growth medium to 1 μ M and 10 nM, respectively. For G1, cells were incubated in 5 mM thymidine for 15 h, allowed to recover in fresh growth medium for 7 h, and incubated with 1 μ M nocodazole for 3 h. Rounded mitotic cells were then harvested by mechanical detachment or knock-off and washed out of nocodazole and allowed to proceed through mitosis and into G1. Adherent cells were harvested by scraping into lysis buffer. For the S phase block, cells were incubated in 5 mM thymidine for 15 h, fresh growth medium for 9 h, and fresh thymidine for an additional 15 h. For the G2 block, an S phase block was performed, and after the second thymidine treatment, fresh medium was added for 5 h. Mitotic cells for Fig. 4 D were prepared by treating cells with 5 mM thymidine for 15 h, allowing cells to recover for 8 h, and then treating cells with 10 nM taxol for 3 h. Rounded mitotic cells were harvested by mechanical detachment.

For C3 treatment, 0.28 μ g GST-C3 (prepared as in Worthylake et al., 2001) per 24-well plate well was incubated in serum-free medium and 1.7 μ l LipofectAmine (Invitrogen) and then added to cells in serum-free medium. Cells were incubated with GST-C3 or GST alone for 90 min, fixed, and processed for microscopy. For Y-27632 treatment, cells were treated with 10 μ M Y-27632 (Welfide Corp.) for 1 h (2 h for rigidity experiments) in growth medium.

Determination of relative cell rigidity

HeLa cells were treated with GST or GST-C3 as for Fig. 1, or with Y-27632 as above. Cells in metaphase of mitosis were identified by phase contrast microscopy (Nikon or Carl Zeiss MicroImaging, Inc.) as having a metaphase plate of condensed chromatin. A glass microneedle was brought laterally to the surface of the cell. Using the micromanipulator (Narishige or Eppendorf) manual control dials, the microneedle shaft was moved laterally to standardized distances (0, 25, 50, 75, 100, and 125 μ m), and images of the cell and the needle tip were captured for each distance of shaft movement, which corresponds to the amount of pressure applied. The location of the microneedle tip against the cell was plotted. Stiffness, or rigidity, is defined as force/change in length. Cell rigidity is plotted as the distance of deformation per amount of force, or pressure, applied. The same needle was used for control and untreated cell measurements within each experiment.

Immunofluorescence and immunoblotting

To visualize actin and DNA, cells plated on glass coverslips were fixed for 10 min in 3.7% formaldehyde in PBS, permeabilized for 5 min in 0.5% Triton X-100 in TBS (150 mM NaCl, 50 mM Tris-Cl, pH 7.6), and washed in TBS containing 0.1% azide. Coverslips were washed in TBS + azide. Cells were labeled with Alexa[®]594 phalloidin (Molecular Probes), Hoechst 33342 (Molecular Probes), or a monoclonal anti-paxillin antibody (BD Biosciences) and an AlexaFluor[®]488 secondary anti-mouse antibody (Molecular Probes). Images were collected using a Carl Zeiss MicroImaging, Inc. Axiophot with a Micromax cooled CCD camera (Princeton Instruments) driven by Metamorph software (Universal Imaging Corp.). We used primary antibodies to RhoA, p190RhoGAP, p120RasGAP, and HRP-conjugated anti-phosphotyrosine (mouse monoclonals; BD Biosciences) and an HRP-conjugated anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories).

RhoA activity assay

The RhoA activity pulldown assay was performed as published (Arthur and Burridge, 2001).

Immunoprecipitations

HeLa cells were treated overnight with 10 nM taxol, and rounded mitotic cells were collected by knock-off (mechanical disruption). The remaining adherent cells comprised the interphase sample. For immunoblotting, coimmunoprecipitation with p120RasGAP, and PP1 treatment, cells were lysed in a modified RIPA buffer (150 mM NaCl, 6 mM Na₂HPO₄, 4 mM NaH₂PO₄, 1% deoxycholic acid, 1% NP-40, 0.1% SDS, 2 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM orthovanadate, 1 mM PMSF, 50 nM calyculin A) and precleared with anti-mouse-conjugated beads (Sigma-Aldrich). Lysates were clarified by centrifugation, protein concentrations were determined and adjusted to equal levels, and lysates were incubated with anti-p190RhoGAP (3 μl/500 μg total protein; BD Biosciences) at 4°C for 1.5 h. Anti-mouse IgG beads were added and incubated for an additional 30 min. Beads were washed three times in lysis buffer and prepared for SDS-PAGE by boiling in sample buffer. For GAP assays, immunoprecipitations were performed as published (Fincham et al., 1999).

PP1 treatment

Sedimented p190RhoGAP on beads from immunoprecipitations, as described above, was washed two times into reaction buffer (50 mM imidazole, pH 7.0, 0.25 mM MnCl₂, 5 mM DTT, 100 mM KCl, 2 mM MgCl₂, 0.2 mg/ml BSA) and divided equally for the following treatments. One sample was left untreated, one sample received PP1 (Calbiochem) alone (1 U), one sample received okadaic acid (final concentration 4 μM) before addition of PP1, and the fourth sample received vanadate (final concentration 2 mM) before PP1. Reactions were incubated at 30°C for 30 min and stopped by the addition of sample buffer and boiling.

GAP activity assay

GAP assays were performed as published (Fincham et al., 1999).

Transfections

Transient transfections were performed with LipofectAmine Plus (GIBCO BRL) according to the manufacturer's instructions.

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