Cell Type-specific Regulation of RhoA Activity during Cytokinesis*

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Rho family GTPases play pivotal roles in cytokinesis. By using probes based on the principle of fluorescence resonance energy transfer (FRET), we have shown that in HeLa cells RhoA activity increases with the progression of cytokinesis. Here we show that in Rat1A cells RhoA activity remained suppressed during most of the cytokinesis. Consistent with this observation, the expression of C3 toxin inhibited cytokinesis in HeLa cells but not in Rat1A cells. Furthermore, the expression of a dominant negative mutant of Ect2, a Rho GEF, or Y-27632, an inhibitor of the Rho-dependent kinase ROCK, inhibited cytokinesis in HeLa cells but not in Rat1A cells. In contrast to the activity of RhoA, the activity of Rac1 was suppressed during cytokinesis and started increasing at the plasma membrane of polar sides before the abscession of the daughter cells in both HeLa and Rat1A cells. This type of Rac1 suppression was shown to be essential for cytokinesis because a constitutively active mutant of Rac1 induced a multinucleated phenotype in both HeLa and Rat1A cells. Moreover, the involvement of MgcRacGAP/CYK-4 in this suppression of Rac1 during cytokinesis was shown by the use of a dominant negative mutant. Because ML-7, an inhibitor of myosin light chain kinase, delayed the cytokinesis of Rat1A cells and because Pak, a Rac1 effector, is known to suppress myosin light chain kinase, the suppression of the Rac1-Pak pathway by MgcRacGAP may play a pivotal role in the cytokinesis of Rat1A cells.

After chromosomal separation at the onset of anaphase, cytokinesis creates two daughter cells endowed with a complete set of chromosomes and cytoplasmic organelles. During this period, cortical actin and myosin II begin to move toward the equatorial region, where they form a contractile cleavage furrow (1–3). Rho family GTPases, which regulate a number of cellular functions including gene expression and cell adhesion (4), also play a pivotal role in cytokinesis (2, 5, 6). Among them, RhoA has been shown to be necessary for cytokinesis in a variety of cell types including Xenopus and sand dollar eggs (7). Furthermore (8, 9), significant progress has been made in the identification of RhoA effectors during cytokinesis (5). One RhoA effector, Rho kinase/ROCK, stimulates myosin II regulatory light chain (MLC) directly by phosphorylation and indirectly by the inhibition of myosin phosphatase (10, 11). Another RhoA effector, citron kinase, also phosphorylates and activates MLC (12). This phosphorylation of MLC is believed to lead to actomyosin contractility and thereby to cytokinesis. However, the role of RhoA in cytokinesis may not be identical in adherent cells and in eggs or poorly adherent cells; well adherent NRK and Swiss 3T3 cells undergo cell division in the presence of an inhibitor of Rho, C3 ribosyltransferase, suggesting that cytokinesis may proceed by a RhoA-independent mechanism in some cell types (13).

In addition to RhoA, Rac1, and Cdc42 have also been implicated in the cytokinesis of mammalian cells, based on the appearance of multinucleated cells among cells expressing constitutively active Rac1 or Cdc42 (14, 15). In agreement with this view, it has been speculated that low microtubule density at the equatorial region leads to the suppression of Rac1 (6). This suppression of Rac1 activity down-regulates Pak, a kinase phosphorylated by Rac1 and thereby suppresses MLC. Therefore, the suppression of Rac1 may also be involved in the contraction of the cleavage furrow.

The activity of Rho family GTPases is regulated by the balance between guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs); GEF activates Rho by catalyzing the uptake of GTP, whereas GAP stimulates the GTPase activity of Rho, leading to its inactivation. Molecular and genetic studies have shown that a mammalian Rho GEF, ECT2, and its Drosophila melanogaster ortholog, Pebble (PBL), are required for cytokinesis (17, 18). Recent studies have shown that these GEFs may be particularly important in the determination of the place and timing of cytokinesis (19, 20). In addition to the GEFs, GAPs also appear to play a critical role during cytokinesis. A GAP for the Rho family GTPases, MgcRacGAP/CYK-4, is a component of the central spindle complex, which bundles microtubules in the central spindle (20–23). The inhibition of MgcRacGAP/CYK-4 by mutants or RNA interference inhibits cytokinesis, inducing multinuclei-
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Roles of Rho Family GTPases in Cytokinesis—Cells were infected with recombinant adenoviruses carrying GFP or GFP-C3. Thirty six hours later, the cells were labeled with BrdUrd (Sigma) for 12 h. After fixation with 70% ethanol, the cells were permeabilized with 0.1% Triton X-100, followed by incubation in PBS containing 30 μg/ml DNase I (Roche Diagnostics) for 1 h. BrdUrd incorporated into the nucleus was detected with anti-BrdUrd antibody (BD Biosciences), followed by Alexa 546-conjugated anti-mouse IgG antibody (Molecular Probes, Inc., Eugene, OR). More than 100 cells that were positive for both BrdUrd and GFP were analyzed to identify the multinucleated phenotype. In other experiments, the cells were transfected with pRMR21-derived expression plasmids of Rho family GTPases or pRed-derived expression plasmids, and 48 h later, the cells expressing marker proteins were analyzed to identify the multinucleated phenotype.

Microinjection of C3 Toxin—The cDNA of C3 toxin was inserted into pGEX-6P-2 (Amersham Biosciences). The purification of C3 toxin was performed according to the manufacturer's protocol. Briefly, glutathione-Transferase-fused C3 was purified on a glutathione-Sepharose column from the cell lysates of Escherichia coli expressing pGEX-6P-2. C3 toxin was excised from glutathione S-transferase with PreScission protease, followed by dialysis against PBS. Cells in the metaphase were microinjected with 0.1 mg/ml C3 in PBS or with PBS alone and were analyzed to identify the multinucleated phenotype as described previously (13).

Effect of Kinase Inhibitors on Cytokinesis—Cells were treated with 40 μM Y-27632 (Calbiochem) for 4 h, 40 μM ML-7 (Calbiochem) for 5 min, or 100 μM blebbistatin (Toronto Research Chemicals Inc., North York, Ontario, Canada) for 1 min. Then, in the continuing presence of the inhibitors, cells in metaphase were identified, and such cells were recorded by DIC images created every 30 s in the case of the Rat1A cells and every 1 min in the case of the HeLa cells. Using these DIC images, the diameter of the contractile ring was measured and plotted to obtain the time course. Each time course was fitted to an exponential curve with GraFit software (Erithacus Software Ltd., Horley, UK), by which the maximum velocity and the half-time (τ/2) of cleavage furrow contraction were calculated.

RESULTS AND DISCUSSION

Changes in the Activity of RhoA and Rac1 in Rat1A Cells Progressing from the G2 to the G1 Phase—To examine whether the changes in the activity of RhoA and Rac1 described in HeLa cells could be generalized to other cell types, we monitored spatio-temporal changes in the activities of RhoA and Rac1 by using Rat1A cells progressing from the G2 to the G1 phase, as described previously (28). Briefly, Rat1A cells expressing Rac1-RhoA or Rac1-Rac1 were excited at 440 nm and imaged for CFP and YFP at 475 and 530 nm, respectively. The intensity ratio YFP/CFP was used to represent the FRET efficiency of the probes, which reflects the GTP/GDP ratio on each probe. Because Rac1 probes are regulated in a manner similar to that of authentic GTPases, the FRET value at each pixel of the digital image reflects the activity of the corresponding GTPase (28). Images of differential interference contrast and YFP-tagged actin were also obtained to follow the morphological changes.

At prophase, the activities of RhoA and Rac1 started decreasing, reaching a nadir at telophase, and gradually increased upon exit from the M phase (Fig. 1A). The activities were then averaged for the entire region of each Rat1A cell and compared with those of HeLa cells (Fig. 1B). RhoA activity started increasing at late telophase in Rat1A cells, whereas it did so in anaphase in the HeLa cells. The time course of the change in the activity of Rac1 was very similar between Rat1A cells and HeLa cells. In order to follow changes in activity over a long period of ~16 h, the objective lens was focused on the basal plasma membrane before imaging and was fixed during the...
entire experiment. Thus, upon the rounding of the cells during mitosis, the images always became out of focus. To obtain clearer images, we looked for cells that had entered into mitosis, and we acquired their images by continuously focusing the lens at the middle depth of the cells (Fig. 2A). The progression of cytokinesis was followed by measuring the breadth of the cells at the cleavage furrow. The averaged RhoA activity increased, whereas the constriction proceeded in HeLa cells, and in Rat1A cells, RhoA activity reached a nadir in the late phase of cytokinesis, slightly before the appearance of the mitotic midbody. We performed similar experiments with NRK and NIH3T3 cells and found that NRK cells behaved in a manner similar to that of HeLa cells, whereas NIH3T3 cells behaved in a manner similar to that of Rat1A cells (Fig. 2B). These observations suggest that the role of RhoA in cytokinesis is cell type-specific.

The changes in the activity of Rac1 were indistinguishable among HeLa, Rat1A, NRK, and NIH3T3 cells (Fig. 3). Suppression of Rac1 activity was most prominent at the cleavage furrow, and the increase in activity was initiated at the polar ends of the plasma membrane in both cell types.

Inhibition of Cytokinesis of HeLa and NRK Cells but Not of Rat1A and NIH3T3 Cells by C3—The lack of increase in RhoA activity during cytokinesis suggested its dispensability in the cytokinesis of Rat1A cells and NIH3T3 cells. To address this issue, we examined the effect of C3 toxin on cytokinesis. The effectiveness of C3 toxin delivered by a recombinant adenovirus was first confirmed by the loss of actin stress fibers in both HeLa and Rat1A cells. To exclude cells that were G1-arrested by the inactivation of Rho family GTPases (31), we stained the cells with BrdUrd, and we determined the number of multinucleated cells among those positive for both BrdUrd and GFP (Fig. 4A). The expression of GFP-C3 significantly induced the multinucleated phenotype in more than 50% of the HeLa cells but only in 10% of the Rat1A cells and 18% of the NIH3T3 cells. We could not perform a similar experiment by using NRK cells because of the toxicity of the recombinant adenovirus carrying GFP-C3 to this cell type. Therefore, we examined the effect of C3 by microinjecting purified C3 toxin into the cytoplasm at metaphase (Fig. 4B). Cytokinesis was remarkably inhibited in HeLa and NRK cells and was slightly disturbed in Rat1A cells by this manipulation. These results suggest that the requirement of RhoA for cytokinesis was less flexible in

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Fig. 1. The activity of Rho family GTPases in HeLa cells progressing from the G2 to the G1 phase. A. Rat1A cells were infected with recombinant adenoviruses for the expression of Raichu-RhoA and Raichu-Rac1, as indicated at left. CFP, YFP, and DIC images were obtained every 1 min with a time-lapse epifluorescent microscope. A ratio image of YFP/CFP was used to represent the FRET efficiency. The stages of the cell cycle were determined by the DIC images. Representative FRET images are shown at each stage of the cell cycle denoted at the top of the figure. The upper and lower limits of the ratio range are shown at the right of each panel. At least four similar images were obtained for each probe, and a representative image is used here. B. in the Rat1A cells from the images in A, the net intensities of YFP and CFP in each cell were measured in order to calculate the averaged YFP/CFP emission ratio. The HeLa cell experiments were performed in essentially the same manner as the Rat1A cell experiments. Because the basal level of the emission ratio varies from cell to cell, the relative emission ratio to that of the G2 phase is used as an arbitrary unit (a.u.). As a control, we used Raichu-Pak-Rho as described (28).
HeLa and NRK cells than in Rat1A cells and NIH3T3 cells. O’Connell et al. (13) have shown that the microinjection of C3 inhibits cytokinesis in HeLa cells but not in NRK cells and Swiss 3T3 cells. The discrepancy regarding the effects on NRK cells may have been due to the difference in the origin of NRK cells, the culturing conditions, or the concentration of C3. In any case, the effect of C3 on cytokinesis appeared to be dependent on the cellular context.

Role of the Suppression of Rac1 Activity in Cytokinesis—Next, we addressed the role of the suppression of Rac1 during cytokinesis. To this end, we expressed constitutively active or dominant negative mutants of Rac1 (Fig. 5A). The expression of Rac1-G12V significantly increased the number of multinucleated cells in both HeLa and Rat1A cells. This observation agreed with the results of previous reports, i.e. it was found that constitutively active Rac1 induced multinucleated cells in HeLa and porcine aortic endothelial cells (14, 15). In contrast to the constitutively active mutant, Rac1-T17N did not increase the number of multinucleated cells to a detectable level. This observation again agreed with those of previous reports (2, 21) showing that a loss of the function of Rac1 did not inhibit cytokinesis.

Among many effector molecules of Rac1, Pak may be involved in the regulation of cytokinesis. Microinjection of Pak inhibits cleavage furrow ingression of Xenopus egg (32). Pak phosphorylates and thereby inhibits MLCK (16, 33), which is known to promote cytokinesis through phosphorylation of myosin II (34). We found that expression of a constitutively active Pak1 mutant, Pak-T423E, significantly increased the number of multinucleated cells both in HeLa and NIH3T3 cells (Fig.
Thus, a decrease in Rac1 activity and the resulting suppression of Pak1 appear to be essential steps of cytokinesis in a variety of cell types.

Inhibition of Cytokinesis by Dominant Negative Mutants of Ect2 and MgcRacGAP/CYK-4—We further studied the mechanism of changes in the activity of Rho family GTPases during cytokinesis. For this purpose, we utilized dominant negative mutants of Ect2 and MgcRacGAP/CYK-4, which have been shown to regulate cytokinesis (18, 22, 23, 25). In cells expressing the dominant negative mutant of Ect2, Ect2-N, the increase in RhoA activity was suppressed at the cleavage furrow but not at the plasma membrane of polar sides (Fig. 6A). This observation agrees with the previously demonstrated recruitment of Ect2 to the cleavage furrow during cytokinesis (18) and suggests that multiple Rho GEFs are activated during cytokinesis. In cells expressing the dominant negative mutant of Ect2, Ect2-N, the increase in RhoA activity was suppressed at the cleavage furrow but not at the plasma membrane of polar sides (Fig. 6A). This observation agrees with the previously demonstrated recruitment of Ect2 to the cleavage furrow during cytokinesis (18) and suggests that multiple Rho GEFs are activated during cytokinesis. In cells expressing the dominant negative mutant of Ect2, Ect2-N, the increase in RhoA activity was suppressed at the cleavage furrow but not at the plasma membrane of polar sides (Fig. 6A). This observation agrees with the previously demonstrated recruitment of Ect2 to the cleavage furrow during cytokinesis (18) and suggests that multiple Rho GEFs are activated during cytokinesis.

In cells expressing the dominant negative mutant of MgcRacGAP, MgcRacGAP-RA, Rac1 activity was not decreased at the cleavage furrow of HeLa cells, indicating that the suppression of Rac1 activity during cytokinesis was primarily mediated by the recruitment of MgcRacGAP (Fig. 6B).

To determine the effect of these mutants on cytokinesis quantitatively, we scored the number of multinucleated cells in the presence or absence of GFP-Ect2-N or MgcRacGAP-RA (Fig. 6C). Both GFP-Ect2-N and MgcRacGAP-RA increased the number of the multinucleated cells in HeLa cells. The effect of these mutants on Rat1A cells was marginal in this assay.

Therefore, to examine the effect of these mutants on cytokinesis more directly, we measured the diameter of the contractile ring as shown by DIC images created during cytokinesis, and we calculated the maximum velocity of its shortening and the half-time of cytokinesis. The half-life of the shortening of the contractile ring was 7.2 min in the HeLa cells and 4.7 min in the Rat1A cells (Fig. 6D). In the presence of GFP-Ect2-N and MgcRacGAP-RA, the half-life of the shortening of the contractile ring was increased to 9.6 and 11.2 min, respectively, in HeLa cells (Fig. 6E). In Rat1A cells, however, only MgcRacGAP-RA increased the half-life of the contractile ring shortening to 7.1 min. These observations suggest the following conclusions. First, the increase in RhoA activity at the cleavage

![Figure 5](http://www.jbc.org/)

**FIG. 5.** Role of Rac1 and Pak1 on cytokinesis. A, HeLa cells, Rat1A cells, NRK cells, and NIH3T3 cells were transfected with constitutively active or the dominant negative mutant of GFP-Rac1 and were analyzed to identify the multinucleated phenotype as in Fig. 4. B, cells were transfected with a plasmid encoding constitutively active Pak1, Pak1 T423E, and analyzed for the multinucleated phenotype. Cont, control.

**FIG. 6.** Effect of dominant negative mutants of Ect2 and MgcRacGAP/CYK-4. A, HeLa cells expressing Ect2-N and Raichu-RhoA were imaged as in Fig. 2A. B, HeLa cells expressing MgcRacGAP-RA and Raichu-Rac1 were imaged as in Fig. 3A. C, HeLa cells and Rat1A cells were transfected with Ect2-N and MgcRacGAP-RA and were analyzed to identify the multinucleated phenotype as in Fig. 4. D, time-lapse analysis of cytokinesis in HeLa and Rat1A cells. The diameter of the contractile ring was measured as illustrated in the left panel. The right panel shows the aligned time courses of cleavage furrow constriction in control cells (n = 10). E, HeLa and Rat1A cells were mock-transfected or transfected with pEGFP-C1-Ect2-N4 or pEredMit-MgcRacGAP-RA and were observed for cytokinesis. Cont., control. The averaged time courses are shown (n > 4).
furrow of HeLa cells was primarily mediating Ect2. Second, the decrease in Rac1 activity at the cleavage furrow was primarily mediated by MgcRacGAP. Third, the activation of RhoA was not essential for the cytokinesis of Rat1A cells. Fourth, in Rat1A cells, suppression of Rac1 plays a more critical role in cytokinesis than activation of RhoA.

Recently, it has been shown that increases in RhoA activity are responsible for cortical rigidity (35). Diffuse increases in RhoA activity at the plasma membrane may play a role in this increased cortical rigidity. However, the results obtained with GFP-Ect2-N seem to indicate that such an increase at the cortex is not sufficient for cytokinesis, unless it is accompanied by an increase in RhoA activity at the cleavage furrow. More importantly, these results demonstrated that the inhibition of cytokinesis by C3 or GFP-Ect2-N in each cell type was closely correlated with an increase in RhoA activity during cytokinesis, as observed by the Raichu probes used here.

Notably, our observations are not necessarily in conflict with the recent finding that MgcRacGAP/CYK-4 phosphorylated by AurA B acts on RhoA at the time of the abscession of daughter cells (24, 25). Because of the limitation in the resolution of the FRET images, we were unable to conclude whether or not RhoA was suppressed at the spindle midbody at the time of abscession.

Role of ROCK and MLCK on the Cytokinesis of HeLa and Rat1A Cells—To understand further the role of Rho family GTPases in the cytokinesis of HeLa and Rat1A cells, we tested the effects of various inhibitors that have been shown to disturb cytokinesis. First, we tested the effects of blebbistatin, an inhibitor of myosin II (36), because cytokinesis can proceed in a myosin II-independent manner in Dictyostelium discoideum (37). As shown in Fig. 7, blebbistatin abrogated the cytokinesis of both HeLa and Rat1A cells. Thus, we proceeded to examine the contribution of ROCK and MLCK, which can induce actomyosin contraction by the phosphorylation of the light chain of myosin II (2). To this end, we treated the cells with Y27632, an inhibitor of ROCK, or ML-7, an inhibitor of MLCK. In HeLa cells, the effect of ML-7 was insignificant, whereas Y-27632 markedly delayed the velocity of cleavage furrow ingression, as reported previously (38). In contrast, ML-7, but not Y27632 significantly inhibited the cleavage furrow ingression of Rat1A cells. Therefore, a relief of Pak suppression seemed to lead to MLCK-promoted cytokinesis in Rat1A cells, as suggested previously (6). One of our co-authors (39) has recently reported an essential role of MLCK in the normal spindle morphology and chromosomal alignment of mitotic HeLa cells by using dominant negative mutants of MLCK. In these HeLa cells expressing the dominant negative MLCK mutants, the frequency of the multinucleated cells was up to 11%, whereas the percentage of multinucleated cells exceeded 50% in the presence of C3 (Fig. 4). In addition, the effect of the dominant negative mutants of MLCK on the velocity of cleavage furrow ingression was markedly weaker than that of Y-27632 (supplemental figure). Therefore, we concluded that the effect on MLCK was prominent in metaphase but less remarkable during cytokinesis in HeLa cells. Altogether, cytokinesis of HeLa cells seems to be more resistant to the inhibition of MLCK than Rat1A cells.

In conclusion, this study revealed that there are at least two pathways leading to actomyosin contraction and the resulting constriction of the cleavage furrow. The contribution of each pathway may depend on the cellular context. For example, the Ect2-RhoA-ROCK pathway is predominant in HeLa cells, whereas the MgcRacGAP/CYK-4-Rac1-Pak-MLCK pathway plays a more important role in the cytokinesis of Rat1A cells. Such differences in the mechanism of cytokinesis may in turn have generated the observed discrepancy in the requirement of RhoA for cytokinesis.

Fig. 7. Differential role of ROCK and MLCK on the cytokinesis of HeLa and Rat1A cells. A, HeLa and Rat1A cells untreated or treated with Y-27632, ML-7, or blebbistatin were observed for cytokinesis, as described in Fig. 6. The averaged time courses are shown (n > 6). Cont., control. B, each time course was fitted to an exponential curve with GraFit software, by which the maximum velocity and the half-time (t1/2) of cleavage furrow contraction were calculated; the results are shown as the mean ± S.D. *p < 0.005 relative to the control.

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Note Added in Proof—Following acceptance of this manuscript, a complementary study was published that provides further genetic support for our proposal that MgcRacGAP/CYK-4/RacGAP50C suppresses Rac during cytokinesis (D’Avino, P. P., Savoian, M. S., and Glover, D. M. (2004) J. Cell Biol. 166, 61–71).

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