Accumulation of GTP-bound RhoA during Cytokinesis and a Critical Role of ECT2 in This Accumulation*

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Running title: *ECT2 as a main exchanger for RhoA activation in cytokinesis*
Summary

We developed a new pull-down assay for GTP-Rho, and examined its level during cell cycle. HeLa cells were arrested in the S phase by thymidine and were enriched in the prometaphase, metaphase, telophase and G1 phase by collecting at 0, 45, 90 and 180 min after the release from the nocodazole arrest, respectively. The level of GTP-Rho did not change significantly from the S phase to the prometaphase, but increased thereafter, peaking in the telophase, and returned to the original level in the G1 phase. The GDP-GTP exchange activity for Rho measured in cell lysates in parallel increased also during the mitosis with a peak in the metaphase. Using this system, we examined a role of ECT2, an exchanger for Rho GTPases, suggested to be involved in cytokinesis (Tatsumoto, T., Xie, X., Blumenthal, R., Okamoto, I., and Miki., T. (1999) J. Cell. Biol., 147, 921-927). Expression of the dominant negative form of ECT2 completely suppressed both the rise of GTP-Rho in the telophase and the increased GDP-GTP exchange activity in the mitotic cell extracts. These results suggest a critical role of ECT2 in Rho activation during cytokinesis.
The small GTPase Rho cycles between the GDP-bound inactive form and the GTP-bound active form, and works as a switch in several cellular processes including stimulus-induced cell to substrate adhesion, motility, and transcriptional activation (1, 2). Rho is also involved in cell cycle progression. Treatment of interphase cells with botulinum C3 exoenzyme arrests the cell cycle progression in the G1 phase, and, conversely, expression of Val14-Rho stimulates the progression from the G1 to S phase (3, 4). Rho appears to exert this action by regulation of cyclin kinase inhibitors (5, 6), probably through an anchorage-dependent mechanism (7). Another action of Rho in the cell cycle is in cytokinesis. Microinjection of either C3 exoenzyme or Rho guanine nucleotide dissociation inhibitor into fertilized eggs of sea urchin or Xenopus embryos inhibited cytokinesis without an effect on nuclear division, resulting in production of multinucleated cells (8, 9, 10). Furthermore, treatment with C3 exoenzyme of cells undergoing cleavage induced regression of the cleavage furrow and reversed the cytokinesis (8). These findings suggest that Rho is activated during or after the nuclear division and this activation is required for induction and maintenance of the cytokinesis.

The above diverse actions of Rho indicate that activation of Rho occurs in both stimulus- and context-dependent manners. This activation has been analyzed by two ways; one is to identify molecules and mechanisms involved in the GDP-GTP exchange of Rho, and the other is to monitor Rho activation by measuring the level of GTP-bound Rho. Exchange of GDP with GTP on Rho is catalyzed by Rho-specific guanine nucleotide exchange factors (GEFs)\(^1\). Several GEFs specific for Rho have already been identified and are characterized by the presence of DH and PH domains (11). Recently, one of them, p115 Rho-GEF, was found to be stimulated by binding to \(G_{\alpha 13}\) (12). Because \(G_{13}\) is coupled to the receptor for lysophosphatidic acid (13), a well-known extracellular stimulus for Rho (14), this finding clarified one of the stimulus-dependent activation mechanisms of Rho. As for measurement of the level of GTP-Rho, Ren et al. (15) developed a pull-down assay using the RBD of rhotekin, one of the Rho effectors.

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binding selectively to GTP-Rho (16). Using this assay, they showed induction of Rho activation by cell adhesion to fibronectin and its enhancement by serum. Similar pull-down assays have been utilized to assess Rho activation in other stimulus-induced processes (17, 18).

Thus, the stimulus-dependent activation of Rho has been verified by the pull-down assays and its mechanisms are being clarified. On the other hand, how the level of GTP-Rho is regulated and changes during the cell cycle remains largely unknown. It is known that cells change their shapes during the cell cycle, being flat in interphase, round in mitosis and cleaved. Whether activation and inactivation of Rho correlate to such shape changes is an interesting question. In the present work, we have developed a new pull-down assay for GTP-Rho, and measured the level of GTP-Rho during the cell cycle. We have also examined the role of ECT2, a Rho-GEF recently found to be involved in the cytokinesis (19), in accumulation of GTP-Rho in mitotic cells.
EXPERIMENTAL PROCEDURES

Plasmids--pCEV32F-ECT2-F encoding the full-length human ECT2 and pCEV32F-ECT2-C encoding the C-terminus of ECT2 were described previously (19). ECT2-N1 and ECT2-N2 were constructed as follows. The region encoding ECT2-N1 (aa 1-335) was amplified by PCR using 5'-CGGGATCCATGGCTGAAAATAGTGTA-3' as a forward primer and 5'-CGGGATCCACTGATTTCTTGAGCTCA-3' as a reverse primer. After amplification, this cDNA fragment was digested with BamHI, and subcloned into pBluescript SK(+) and sequenced. After sequencing, this clone was digested with BamHI and EcoRI, and cloned into pCEV32F (20). For construction of ECT2-N2, the ECT2-F was digested by BstEII and self-circularized. pEXV-myc-Val14RhoA and pCMV-myc-Asn19RhoA were described previously (21). pGEX-3X-RhoA was provided by Y. Takai, and expressed as described (22).

pGEX-3X-mouse rhotekin RBD (aa -8-89) (16), pGEX-4T-1-mouse mDia RBD (aa -2-304) (23), and pGEX-4T-3-mouse ROCK-II RBD (aa 800-1137) (18) were described previously. pGEX-6P-3-mouse citron RBD (24) was constructed as follows. The region encoding mouse citron RBD (aa 1124-1286) cDNA was amplified by PCR using 5'-CGACGTACAGAAGAAGCACGCCATGCTGG-3' as a forward primer and 5'-CGATGCGGCCGCCTACGGGTGGTCCGTGGCTTTGC-3' as a reverse primer. This fragment was digested with BsiWI and NcoI, and cloned into similarly cut pEGFP-C1. This plasmid DNA and pGEX-6P-3 were digested with BamHI and EcoRI, and ligated. These pGEX plasmids were introduced in E.coli BL21(DE3)pTrx (25), and GST-fusion proteins were expressed and purified by glutathione-coupled Sepharose 4B beads (Pharmacia).

Cell Culture, Cell Cycle Synchronization and Transfection--HeLa cells were cultured to semi-confluency in DMEM supplemented with 10% FCS. Cells were trypsinized and one-twentieth of the culture was seeded on a new 10-cm culture dish. After one day in DMEM containing 10% FCS, cells were synchronized in the S phase with the double-thymidine block method (26). In brief, cells were cultured in DMEM containing 5% FCS
and 10 mM thymidine for 15 h, incubated in fresh DMEM containing 10% FCS for 9 h and then cultured again in the thymidine-containing medium for 15 h. In some experiments, we exchanged the medium with serum-free Opti-MEM (Gibco-BRL) at 3 h in the second thymidine block, and transfected cells with pCEV32F plasmid encoding either ECT2-F, ECT2-N1, ECT-N2 or ECT2-C with Lipofectamine (Gibco-BRL) for 3 h. After transfection, the cells were cultured again in the thymidine-containing medium for another 9 h. Cells synchronized in the S phase were then cultured in fresh DMEM containing 10% FCS. After 6 h, nocodazole was added at the final concentration of 40 ng/ml, and the culture was continued for another 6 h. Round mitotic cells enriched by this procedure were further purified by shake-off procedure. Collected cells were suspended in fresh DMEM containing 10% FCS to release from the nocodazole-arrest. At 0, 45, 90 and 180 min after the release, cells were harvested and subjected to analyses.

COS-7 cells were cultured in DMEM containing 10% FCS to semi-confluency in a 10-cm dish, and transfected with 6 µg of pEXV-myc-Val^{14}RhoA or pCMV-myc-Asn^{19}RhoA with Lipofectamine as described above. After 24 h, the cells were washed with PBS twice before lysis.

**Pull-down Assay for GTP-Rho**--Cells were lysed in 50 mM Tris-HCl (pH 7.5) containing 100 mM NaCl, 1 mM EDTA, 5 mM MgCl_{2}, 10% glycerol, 50 mM NaF, 1 mM Na_{2}VO_{4}, 1 mM dithiothreitol, 50 µg/ml phenylmethylsulfonyl fluoride, 10 µg/ml each of leupeptin and pepstatin and NP-40 (1% for COS cells and 0.1% for HeLa cells) (lysis buffer). Cell lysates were clarified by centrifugation at 30,000 x g at 4°C for 20 min, and the supernatants were incubated with 30 µg GST-RBD fusion conjugated with glutathione-beads at 4°C for 2 h. The beads were washed twice with lysis buffer, and subjected to SDS-polyacrylamide gel electrophoresis on a 12% gel. Bound RhoA was detected by Western blot using a monoclonal antibody against RhoA (Santa Cruz Biotechnology).

**GDP-GTP Exchange Assay for Rho**--[^{3}H]GDP-bound RhoA was prepared by incubating 10 pmol of GST-RhoA with 4 µM[^{3}H]GDP (15,000 cpm/pmol) (Amersham Pharmacia
Biotech) in 20 mM Tri-HCl (pH 7.5) containing 10 mM EDTA, 5 mM MgCl₂ and 0.24% CHAPS in a total volume of 24 µl for 20 min at 30°C. One µl of 0.375 M MgCl₂ was added to the reaction mixture to stop the reaction. The GDP-GTP exchange activity was assayed by incubating [³H]GDP-bound GST-RhoA and cell lysates as described previously (27).

**Immunofluorescence Microscopy**—HeLa cells were either cultured on a coverglass (the S-phase cells) or dissociated and attached to a poly-L-lysine-coated coverglass (the mitotic cells). Fixation, permeabilization and incubation were carried out as described previously (19). Monoclonal anti-β-tublin antibody (SIGMA) and polyclonal anti-FLAG antibody (Santa Cruz Biotechnology) were used as primary antibodies at 1/200 dilution, and Alexa Fluor™ 488-labeled goat anti-rabbit IgG and Alexa Fluor™ 488-labeled goat anti-mouse IgG antibodies (Molecular Probes) as secondary antibodies at 1/200 dilution. DAPI was used for staining of the nucleus. Images were obtained using a Zeiss Axiophot fluorescence microscope.
RESULTS

We previously identified several Rho effectors including ROCK-I and -II (28, 29), p140mDia (30), citron (24) and rhotekin (16). These effectors selectively bind the GTP-bound form of Rho. Among them, the RBD of rhotekin and that of ROCK-II have been used in a pull-down assay for GTP-Rho (15, 17, 18). To compare their binding potencies with RBDs of other Rho effectors, we expressed the RBD of each effector as a GST fusion protein. Each GST fusion was then incubated with lysates of COS cells expressing Val14-RhoA, and RBD-bound Rho was recovered by precipitation with glutathione-beads. As shown in Fig. 1A, only the mDia-RBD could precipitate a significant amount of Val14-RhoA, while the amounts precipitated by other RBDs including rhotekin were negligible. Quantitative analysis indicated that about 5% of Val14-Rho expressed in COS cells was precipitated by mDia-RBD under the present assay conditions. The binding of Val14-Rho to mDia-RBD appeared dependent on its GTP-bound state, because no precipitation of Rho was found when COS cell lysates containing Asn19-Rho were subjected to this assay (Fig. 1B).

We then used this pull-down assay, and examined a change in the level of GTP-Rho during the cell cycle. HeLa cells were synchronized as described under "Experimental Procedures", and cells arrested in the S phase by thymidine or at 0, 45, 90 and 180 min after the nocodazole release were collected. Analysis of cell morphology (Fig. 2A) indicated that 98% of cells collected at 0 min of the nocodazole release were in prometaphase (panel 2), 85, 10 and 5% of cells collected at 45 min in metaphase, anaphase and prometaphase respectively (panel 3), 90% of cells collected at 90 min in telophase (panel 4) and 98% of cells after 180 min in the G1 phase (panel 5). When these cell populations were subjected to the pull-down assay, we detected the significant amount of GTP-Rho in the S-phase cells (Fig. 2B). The level of GTP-Rho did not significantly change when cells entered the M phase and were arrested in the prometaphase with nocodazole. However, following the nocodazole release, the level of GTP-Rho increased at 45 min in the metaphase and reached the maximum at 90 min in the
telophase. It then decreased as the cells entered the G1 phase, but the level at the early G1 phase appeared to be higher than that found in the S phase. These results suggest that RhoA is activated extensively in mitosis. To examine if this activation is due to activation of the GDP-GTP exchange activity, the GEF activity for Rho was examined in lysates of these cells by measuring the rate of $[^3H]$GDP dissociation from RhoA (Fig. 2C). The GEF activity was low in the lysates of S-phase cells, was significantly enhanced in mitotic cells at 0 min, peaked at 45 min after the nocodazole release in the meta- to anaphase and slightly decreased at 90 min in the telophase.

We next examined the role of ECT2 in the accumulation of GTP-Rho and in the increase in the GEF activity in mitotic cells. ECT2 and its Drosophila homolog, Pebble, have recently been suggested to be involved in cytokinesis (19, 31). The full-length ECT2 (ECT2-F), two C-terminally truncated mutants, ECT2-N1 and ECT2-N2, and one N-terminally truncated mutant, ECT2-C (Fig. 3A), were transiently expressed individually in synchronized cells, and the effects on cytokinesis were examined. While ECT2-F as well as ECT2-C did not show any effect, expression of ECT2-N1 and ECT2-N2 induced the production of multinucleated cells as described (19). Of the two, ECT2-N1 showed stronger effects, causing about 20% of cells expressing this mutant multinucleate (Fig. 3B). We then used the pull-down assay and analyzed the level of GTP-Rho in ECT2-N1-expressing cells during mitosis. As shown in Fig. 3C, the accumulation of GTP-Rho at 90 min after the nocodazole release was almost completely abolished by this expression. On the other hand, the level of GTP-Rho at 0 and 180 min did not change from the control cells. Consistent with this finding, the activity of GDP-GTP exchange for Rho was comparable between the control and ECT2-N1-expressing cells at 0 min after the nocodazole release, but its increase at 45 and 90 min was suppressed by ECT2-N1 expression (Fig. 3D). A previous study (19) showed that phosphorylation of ECT2 occurs in mitosis and that this phosphorylation activates the exchange activity of this molecule. To examine whether expressed ECT2-N1 suppressed the ECT2 phosphorylation, we probed lysates of control cells and cells expressing ECT2-
N1 with anti-ECT2 antibodies. Expression of ECT2-N1 affected neither the level of endogenous ECT2 nor its phosphorylation upon the entry of cells to the M phase (Fig. 4). The mobility shift of ECT2 was already found in the prometaphase (at 0 min), and continued to be present at 45 min but, interestingly, was not seen at 90 min.
DISCUSSION

In this work, we used the pull-down assay and revealed the extensive accumulation of GTP-Rho during the mitosis. Our assay to pull down GTP-Rho uses the RBD of mDia. We compared potencies of RBDs of several Rho effectors for precipitating GTP-Rho from the same cell lysates, and found that the precipitation by mDia-RBD was most sensitive and reproducible under our assay conditions. One RBD that was not tested in this study is the C-terminus of phospholipase D1. This domain was also found to pull down GTP-Rho in vitro, although, unlike mDia, it appears to be able to interact with other members of Rho GTPases (32). Using the mDia assay, we found that the level of GTP-Rho increased from the metaphase and peaked at the telophase. This time course is consistent with the suggested action of Rho to induce and maintain the cytokinesis. Compared with this accumulation, the levels of GTP-Rho in the G1 and S phase were relatively low, suggesting that the actions of Rho in these phases are mediated by relatively small populations of GTP-Rho generated transiently and locally at its site of actions. We did not see the complete loss of GTP-Rho in the transition of the S to M phase. This result may suggest that cell rounding associated with the entry to the M phase is not regulated by the level of GTP-Rho.

Consistent with the above findings, we found the increased Rho-GEF activity in lysates of cells in the M phase. However, this increase did not parallel with the GTP-Rho accumulation, but preceded it. This discrepancy raises several possibilities. One is that the catalytic activation and the action in the cell of GEF may occur at consecutive but separate steps, the latter being, for example, regulated by intracellular targeting. It may also well be that the high level of GTP-Rho is caused by down-regulation of GAP activity. Another interesting possibility is that GTP-Rho generated during mitosis binds to Rho effectors involved in cytokinesis, and are stabilized by this binding. In this case, GTP-Rho may work as a structural component. The presence of Rho in the contractile ring and in the midbody was already reported (33, 34).

Finally, we found that the expression of a dominant negative ECT2 almost completely...
inhibited the increase of the Rho-GEF activity in mitosis and suppressed the GTP-Rho accumulation in the telophase. These results suggest that ECT2 is a main GEF working in this process. These effects of ECT2-N1 appear surprising, given that this expression caused only 20% of the total cell population multinucleate. However, this percentage was seen after the cell division, indicating that about 40% of cells failed to divide. We further found that the number of multinucleate cells increased on subsequent divisions. These findings suggest that most of transfected cells expressed this mutant protein to some extent. Notably, the GTP-Rho level at 0 and 180 min was not different between the control and ECT2-N1 expressing cell populations, indicating that the generation of GTP-Rho in these phases is carried out by GEF(s) different from ECT2. Tatsumoto et al. (19) previously found the M-phase-associated phosphorylation of ECT2, and reported the activation of its exchange activity by phosphorylation. We confirmed it in this work. We further found that the phosphorylation was almost complete in the prometaphase (at 0 min), when little accumulation of GTP-Rho was found. This may imply that in order for ECT2 to act as a Rho-GEF in the cell, an additional step is required after the phosphorylation, as discussed above. This possibility is suggested also by the experiment with a dominant negative ECT2-N1, of which expression did not have any effect on the phosphorylation. It is interesting in this respect to test whether the expression of ECT2-N1 affects the localization of endogenous ECT2 during mitosis. ECT2-N1 was localized in the midbody in weakly-expressing cells as did endogenous ECT2 (19) (data not shown). An additional finding in the phosphorylation experiment is that the phosphorylation disappeared in cells in the telophase at 90 min after the nocodazole release, a time at which the extensive accumulation of GTP-Rho was observed. This may suggest again a possibility of the binding and stabilization of GTP-Rho in the contractile ring. Another possibility is that binding of some component(s) to ECT2 during the mitosis activates its exchange activity without phosphorylation. Alternatively, ECT2 may trigger the cascade of exchanger activation. Theses issues should be clarified in future studies.
Acknowledgment

The authors thank K. Fujisawa, T. Ishizaki, N. Watanabe and M. Maekawa for helpful discussions, and T. Arai and H. Nose for secretarial assistance. K. T is a recipient of the fellowship from the Organization for Pharmaceutical Safety and Research.
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Footnotes

*This work was supported in part by a Grant-in-Aid for Specially Promoted Research from the Ministry of Education, Science, Culture and Sports of Japan, and grants from the Organization for Pharmaceutical Safety and Research and the Human Frontier Science Program.

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The abbreviations used are; GEF, guanine nucleotide exchange factor; DH, Dbl. homology domain; PH, pleckstrin homology domains; DMEM, Dulbecco’s modified Eagle medium; FCS, fetal calf serum; PBS, phosphate-buffered saline; DAPI, 4’,6-diamidino-2-phenylindole; GAP, GTPase-activating protein; RBD, Rho-binding domain
Figure legends

Fig. 1. Selective precipitation of the GTP-bound RhoA by GST-RBD of mDia. A, lysates of COS cells expressing myc-tagged Val14-RhoA (500 µg protein) were incubated with 30 µg each of GST-RBD of ROCK-II (lanes 1 and 2), mDia (lanes 3 and 4), rhotekin (lanes 5 and 6), or citron (lanes 7 and 8) or with GST (lanes 9 and 10) conjugated with glutathione-Sepharose 4B beads. After 2 h at 4˚C, the suspension was centrifuged and Val14-RhoA in the supernatants (lanes 1, 3, 5, 7, and 9) and the precipitates (lanes 2, 4, 6, 8, 10) were detected by Western blot analysis using anti-myc antibody as descried under "Experimental Procedures". B, lysates of COS cells expressing either Asn19-RhoA (lane 1) or Val14-RhoA (lane 2) were subjected to the pull-down assay using mDia-RBD, and precipitated RhoA was determined by Western blot analysis.

Fig. 2. Cell cycle-associated changes in the level of GTP-Rho and the activity of GDP-GTP exchange of RhoA. A, cell morphology. HeLa cells were synchronized by the use of thymidine and nocodazole as described under "Experimental Procedures". Cells arrested in the S-phase (panel 1), collected at 0 (panel 2), 45 (panel 3), 90 (panel 4) and 180 (panel 5) min after the nocodazole release were fixed and stained for β-tublin. B, pull-down assay. Lysates of cells collected as described above were subjected to the pull-down assay, and precipitated RhoA was detected by Western blot analysis (upper panel). The amounts of input RhoA was shown in the lower panel. C, the GDP-GTP exchange activity in vitro. Lysates of cells collected at S phase (●), and at 0 (△), 45 (■), 90 (□) min after the nocodazole release were used for measurement of the GDP-GTP exchange activity for Rho as described under "Experimental Procedures". The basal exchange found with the lysis buffer alone (○) was shown as a control. This result was a representative of three independent experiments.

Fig. 3. Inhibition of the RhoA activation and cytokinesis by expression of a dominant negative form of ECT2. A, diagrams of ECT2 and its truncation mutants. The domain structure of ECT2 was shown by a box, and the full-length ECT2 and truncation mutants
were shown by thick lines below. BRCT-1/2, BRCA carboxyl terminus domains 1 and 2. NLS, nuclear localization signal. B, production of multinucleated cells by expression of ECT2-N1. HeLa cells were transfected with either an empty vector (vector) or pCEV32F-ECT2-N1 and stained with anti-FLAG antibody for detection of expressed protein (left panel) and with DAPI (right panel) for nucleus. Arrowheads indicate multinucleated cells. C, inhibition of accumulation of GTP-Rho by expression of ECT2-N1. Lysates from control HeLa cells (-) and cells expressing ECT2-N1 (+) collected at 0, 90 and 180 min after the nocodazole release were used for the pull-down assay for GTP-Rho, and precipitated Rho were detected by Western blot analysis. D, inhibition of the GDP-GTP exchange activity for RhoA by ECT2-N1. Control HeLa cells (bold lines) or cells expressing ECT2-N1 (broken lines) were similarly treated with thymidine and nocodazole in parallel, and collected at 0 (Δ), 45 (○), 90 (■) min after the nocodazole release. Cell lysates were subjected to the assay for the GDP-GTP-exchange activity as described under "Experimental Procedures". A representative result from three independent experiments was shown.

Fig. 4. Effects of ECT2-N1 expression on phosphorylation of endogenous ECT2. HeLa cells with or without ECT2-N1 transfection were collected, and subjected to the Western blot analysis with anti-ECT2 antibody as described under "Experimental Procedures". Phosphorylation of ECT2 was assessed by the mobility shift on SDS-polyacrylamide gel electrophoresis on an 8% gel.
| Time (min) | S  | 0  | 45 | 90 |
|-----------|----|----|----|----|
| **ECT2-N1** (−) | S  | 0  | 45 | 90 |
| **ECT2-N1** (+) | S  | 0  | 45 | 90 |

The diagrams show the results of the experiment at different time points (0, 45, 90 minutes). The left side represents the negative control (−) and the right side represents the positive control (+).
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J. Biol. Chem. published online April 12, 2000

Access the most updated version of this article at doi: 10.1074/jbc.C000212200

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