Myosin plays an important role in mitosis, especially during cytokinesis. Although it has been assumed that phosphorylation of regulatory light chain of myosin (RLC) controls motility of mammalian non-muscle cells, the functional significance of RLC phosphorylation remains uninvestigated. To address this problem, we have produced unphosphorylatable RLC (T18A/S19A RLC) and overexpressed it in COS-7 cells and normal rat kidney cells. Overexpression of T18A/S19A RLC but not wild type RLC almost completely abolished concanavalin A-induced receptor cap formation. The results indicate that myosin phosphorylation is critical for concanavalin A-induced gathering of surface receptors. T18A/S19A RLC overexpression resulted in the production of multinucleated cells, suggesting the failure of proper cell division in these cells. Video microscopic observation revealed that cells expressing T18A/S19A RLC showed abnormalities during mitosis in two respects. One is that the cells produced abnormal cleavage furrows, resulting in incomplete cytokinesis, which suggests that myosin phosphorylation is important for the normal recruitment of myosin molecules into the contractile ring structure. The other is that separation of chromosomes from the metaphase plate is disrupted in T18A/S19A RLC expressing cells, thus preventing proper transition from metaphase to anaphase. These results suggest that, in addition to cytokinesis, myosin and myosin phosphorylation play a role in the karyokineti

Myosin is a motor protein that binds actin filaments and produces force using the chemical energy of ATP. It is known that actin and myosin undergo reorganizations in cultured mammalian cells in response to cellular signals in various cellular motile processes such as chemotaxis, phagocytosis, capping of surface receptors, and cytokinesis (1–16). For example, actin and myosin form stress fibers in interphase cells, but this structure disappears at the onset of mitosis. Cytokinesis, actomyosin is redistributed into the equatorial region to produce the cleavage furrow (see Refs. 17 and 18 for reviews). On the other hand, a signal initiated by the binding of a lectin to its receptor triggers reorganization of actomyosin thus gathering the cleavage site. This paper is available online at http://www.jbc.org

Effects of the Regulatory Light Chain Phosphorylation of Myosin II on Mitosis and Cytokinesis of Mammalian Cells*

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Myosin is essential for the motility activity in the actomyosin system, so it has been thought that myosin plays a key role in dictating cellular motile processes. For both smooth muscle and non-muscle cells (in contrast to highly differentiated striated muscle cells), actomyosin motor activity is primarily regulated by phosphorylation of the regulatory light chain of myosin (19–21). Myosin regulatory light chain (RLC)1 is phosphorylated at various sites. Serine 19 and threonine 18 are the sites responsible for activation of myosin motor activity, and the former site is thought to be physiologically more important (see Refs. 22–24 for reviews). The protein kinase responsible for phosphorylation at these sites is Ca2+/calmodulin-dependent myosin light chain kinase (MLCK), and in smooth muscle cells, this kinase primarily dictates the level of myosin phosphorylation (see Refs. 22 and 24 for reviews). In non-muscle cells, although MLCK is thought to be important for myosin phosphorylation, other protein kinases also play a role in regulating the phosphorylation of myosin (25–27). On the other hand, phosphorylation at serine 1/serine 2 and threonine 9 of the regulatory light chain, which is catalyzed by protein kinase C and Cdc2 kinase, is inhibitory, and phosphorylation at these sites decreases the affinity for actin (28–30).

The bulk of available evidence supports the conclusion that myosin is a key component in cytokinesis. Immunocytochemistry has revealed that myosin is present at the equatorial region during cell division (12, 13, 16, 31). Inhibition of cytokinesis by microinjection of anti-myosin antibodies further suggests the importance of myosin function in cell division (32). Conclusive evidence was obtained by genetic manipulation, and it was found that the deletion of the gene for myosin II in Dicystelium cells abolishes cytokinesis (33).

A remaining question is whether or not phosphorylation of myosin is required for cytokinesis. Fishkind et al. (34) reported that microinjection of constitutively active MLCK into mitotic cells did not affect the formation of cleavage furrows and cytokinesis, although the transit time from nuclear envelope breakdown to anaphase onset was delayed. However, myosin in mitotic cells is phosphorylated (at least to a certain extent), and injection of active MLCK may not significantly change the phosphorylation level of myosin at the cleavage furrow, as the myosin phosphorylation level increases in non-injected cells at the onset of telophase. Whereas the bulk of evidence supports the idea that myosin II function is critical for cell division, the role of serine 19 phosphorylation during cell division in mammalian cells remains obscure. In the present study, we overex-

1 The abbreviations used are: RLC, regulatory light chain of myosin; CMV, cytomegalovirus; Cy5, indodicarbocyanine; ConA, concanavalin A; DAPI, 4′,6-diamidino-2-phenylindole; GFP, green fluorescent protein; MLCK, myosin light chain kinase; NRK, normal rat kidney; PKC, protein kinase C; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine B isothiocyanate.
pressed a myosin regulatory light chain mutant, T18A/S19A RLC, which eliminated the phosphorylation sites in order to hamper myosin phosphorylation. The regulatory light chain was tagged with green fluorescent protein (GFP) to identify the cells transfected with the RLC-GFP construct. Overexpression of unphosphorylatable RLC in mammalian cells abolished surface receptor cap formation. Furthermore, normal cytokinesis was significantly disrupted by the overexpression of unphosphorylatable RLC in mammalian cells. These results indicate that the phosphorylation of myosin regulatory light chain is critical for cell division of mammalian non-muscle cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction enzymes and modifying enzymes were purchased from New England Biolabs (Beverly, MA). Smooth muscle myosin and MLCK were prepared from frozen turkey gizzards (30, 35). Actin was prepared from rabbit skeletal muscle according to Spudich and Watt (36). *Xenopus* oocyte calmodulin (37) and smooth muscle myosin RLC were expressed in Escherichia coli and purified as described (38, 39).

**Production of Monoclonal Antibody against Phospho-RLC**—A monoclonal antibody against phosphoserine 19 of RLC was prepared, basically according to Yano et al. (40). A phosphopeptide KRKPQRTAYpSVNFAMC (where p indicates phosphoserine) (p-Ser19) coupled to keyhole limpet hemocyanin was used as an antigen.

**Production of GFP-Wild Type and GFP-Mutant Regulatory Light Chain Fusion Constructs**—The cDNA fragment of RLC subcloned into a pT7-7 vector. The entire cDNA insert was then excised and used to transfect *E. coli*.

**Expression of GFP-Chimeric RLC and Biochemical Procedures**—RLC-GFP chimeric proteins were expressed in the BL21 (DE3) *E. coli* strain that has T7 RNA polymerase and was purified as described (38).

**In vitro phosphorylation** was carried out using 20 μg/ml partially purified RLC-GFPs in the presence of 20 μM NaCl, 2 mM CaCl2, 1 mM MgCl2, 1 μM microcystin-LR, 2 mM ATP, and 20 μM Tris-HCl, pH 7.5. The assay contained 10 μg/ml calmodulin with or without 20 μM MLCK. The reaction solutions were incubated for 15 min at 30 °C, and then phosphorylation of RLC-GFP was detected by Western blotting with anti-RLC antibody (Fig. 1B, lanes 1 and 2) followed by Western blots conjugated anti-rabbit antibody (Jackson ImmunoResearch). For visualizing the cap structures and phosphorylated myosin, the cells were treated with ConA as described below and fixed in solution I. The fixed cells were then incubated with anti-anti-phospho–RLC (Ser19) monoclonal antibody followed by indocarbocyanine (Cy5)-conjugated anti-mouse antibody (Jackson ImmunoResearch). Images of labeled cells were acquired with a Leica microscope equipped with a 100-watt Hg arc lamp for epifluorescence microscopy. Cells were viewed with 40 or 100× Nikon (NA 1.3) Planapo objectives with 2.5 or 5× camera eyepiece, and images were projected onto the face of a Photometrics thermoelectrically cooled CCD camera (RCA 501 chip). For data restorations, three-dimensional digital images were flat field-corrected, background-subtracted, and normalized for temporal fluctuations in excitation intensity to constant integrated optical density. Prepared images were then processed with an iterative deconvolution algorithm with non-negativity constraints, using determined point spread function for the microscope to at least partially reverse the blurring introduced by the optics (46).

**Induction of ConA Capping and Determination of Multinuclear Cells**—Transfected cells were incubated with 20% conA-grown mammalian cells (NRK52E) followed by Western blots conjugated anti-rabbit antibody (Jackson ImmunoResearch). The authenticity of the RLC-GFP chimeric proteins was also recognized by anti-RLC antibody (Fig. 1B, lanes B, and C). The assembly was detected with the RLC-GFP construct as a template.

**Results**—The specificity of the GFP signal was determined by an increase in total myosin II phosphorylation. The Western blotting analysis of anti-anti-phospho–RLC antibody (Fig. 1B, lanes B, and C) confirms the specificity of the GFP signal. (A) To determine the number of nuclei in cells, the living cells were stained with Hoechst 33342 (Molecular Probes) at 72 h after the transfection. In order to estimate the difference in the myosin phosphorylation of mutant T18A/S19A RLC expressing cells and wild type RLC expressing cells, the transfected cells were treated with non-tagged ConA as described above and then the cells were fixed as described above. The cells were stained with rabbit polyclonal anti-myosin antibodies (rabbit polyclonal and anti-non-muscle myosin IIb antibodies) was then followed by Cy5-labeled anti-mouse and TRITC-labeled anti-rabbit second antibodies, respectively. The fluorescence intensity was determined with digital fluorescent microscopy using a cooled CCD camera.

**Video Microscopy**—Transfected cells in mitotic phase were identified by GFP signal and the appearance of a metaphase plate with a Nikon (Tokyo, Japan) DIAPHOT 300 inverted fluorescence microscope with a 40× Nikon objective. At 30 min after the release from nocodazole arrest, phase contrast images of the transfected cells were monitored for 3–4 h in culture medium containing 25 μM Hepes, pH 7.2, or L15 medium (Sigma) by time-lapse video microscopy.

**Expression of RLC-GFP Chimeric Protein in Mammalian Cultured Cells**—The authenticity of the RLC-GFP chimeric protein was confirmed by Western blotting experiments. Western blots of partially purified wild type RLC-GFP and T18A/S19A RLC-GFP were probed with anti-anti-phospho–RLC antibody (Jackson ImmunoResearch). The authenticity of the RLC-GFP chimeric proteins was also recognized by the antibody for both wild type and mutant proteins (Fig. 1, *lanes 1 and 2*). The expression of the 47-kDa peptides was not found before isopropyl-1-thio-D-galactopyranoside induction (data not shown). Furthermore, the 47-kDa proteins were also recognized by anti-anti-phospho–RLC antibody (Fig. 1B, lanes B, and C).

**Cell Culture and Synchronization**—Epithelial type NRK cells (NRK52E; supplied by Dr. Yu-Li Wang, University of Massachusetts Medical Center) and COS-7 cells were used in the present study. NRK and COS-7 cells were maintained in Kaighn's modified F-12 medium (Sigma) containing 10% fetal bovine serum, respectively. Transfection of plasmids was carried out by electroporation using a Gene Pulser II (Bio-Rad). Transfected cells were seeded onto glass coverslips and cultured for 72–96 h. Mitotic cells were obtained following 4 h of treatment with nocodazole (NRK52E, 0.75 μg/ml; COS-7, 0.05 μg/ml, respectively).
Myosin II Phosphorylation and Cytokinesis

To examine whether or not GFP-tagged RLC is incorporated into myosin in transfected cells, RLC-GFP chimera was expressed in COS-7 cells, and the localization of the GFP signal was observed. COS-7 cells were transiently transfected with GFP-chimeric RLC constructs as described under “Experimental Procedures.” As shown in Fig. 2, wild type RLC-GFP showed filamentous localization that coincides with the localization of myosin and F-actin (Fig. 2, A, B, E, and F). The result suggests that GFP-tagged RLC is incorporated into myosin molecules in cells and that myosin containing RLC-GFP localized in stress fibers, thus retaining authenticity in intracellular localization. T18A/S19A RLC-GFP also co-localized with myosin and F-actin (Fig. 2, C, D, G, and H), indicating that the mutation at the phosphorylation sites of RLC does not change the resting myosin localization. Cells transfected with GFP alone did not show any filamentous localization, although stress fibers were observed in these cells when probed by Texas Red-conjugated phalloidin staining (data not shown).

Effect of Myosin RLC Phosphorylation on Surface Receptor Capping—It has been known that lectin binding to surface receptors induces significant rearrangement of cytoskeletal structures that in turn results in the aggregation of receptors into clusters, known as “capping” (5–11). Actomyosin function is known to be critical for this process. To investigate the role of myosin RLC phosphorylation during this process, COS-7 cells transfected with either wild type or mutant RLC-GFP were treated with ConA to induce capping of cell surface receptors. The expression level of GFP-RLC was estimated by digital fluorescence microscopy, and cells expressing similar levels of the wild type or mutant GFP-RLC were compared. For COS-7 cells expressing wild type RLC-GFP, normal capping of surface receptors was observed in response to ConA (Fig. 3B). During the cap formation, the wild type RLC-GFP signal was concentrated under the ConA cap suggesting that myosin containing GFP-tagged RLC responded to the ConA-induced signal (Fig. 3A). This suggests that myosin with GFP-tagged RLC undergoes normal reorganization during the ConA-induced capping process. In contrast, ConA-induced receptor cap formation was markedly hampered in T18A/S19A RLC-GFP expressing COS-7 cells (Fig. 3E). In these cells, accumulation of RLC-GFP was not observed, suggesting that unphosphorylatable myosin having T18A/S19A RLC-GFP does not proceed with the reorganization observed for the wild type (Fig. 3D). Immunostaining of untransfected cells as well as wild type RLC-GFP-transfected cells with anti-phospho-RLC antibody revealed that phosphorylated RLC accumulated under the ConA cap (Fig. 3C), also suggesting that myosin phosphorylation is involved in the capping process. At 40 min after treatment with ConA, 76% of the wild type RLC-GFP-transfected cells formed capping structures, whereas less than 28% of the cells transfected with the mutant RLC-GFP were able to form capping structures (Table II). In order to determine the difference in phosphorylation of myosin, the GFP-RLC expressing cells were co-stained with anti-myosin heavy chain antibody and anti-phospho-RLC antibody, and the ratio of these signals was used to estimate the difference in the extent of phosphorylation. After ConA treatment, the cells expressing T18A/S19A RLC-GFP had approximately 50% less RLC phosphorylation than those expressing wild type or non-transfected cells (not shown). The disruption of the cap formation in T18A/S19A RLC-GFP expression is thus explained by a decrease in phosphorylated myosin molecules. Although the decrease is explained by the substitution of the endogenous RLC by T18A/S19A RLC-GFP in myosin molecules, it is also possible that free T18A/S19A...
RLC-GFP inhibits MLCK, thus decreasing the phosphorylation of RLC actually incorporated in myosin molecules. To address this possibility, myosin II was phosphorylated by MLCK in the presence of excess concentrations of free RLC-GFP (Fig. 4). The myosin phosphorylated by MLCK was subjected to SDS-polyacrylamide gel electrophoresis followed by autoradiography. Since RLC-GFP has molecular mass of 47 kDa, the phosphorylation of the intrinsic RLC in myosin molecule is easily distinguished by SDS-polyacrylamide gel electrophoresis. Neither wild type nor T18A/S19A RLC-GFP significantly affected the phosphorylation of native myosin-bound RLC by MLCK, suggesting that free RLC-GFP molecules do not inhibit MLCK to any measurable extent. The results also suggest that a significant number of the myosin light chains in myosin are substituted by RLC-GFP. The present results demonstrate that myosin RLC phosphorylation is critical for gathering surface receptors into cap structures, indicating that phosphorylation of RLC plays an essential role in non-muscle myosin function in capping of surface receptors.

\[ \text{RLC-GFP inhibits MLCK, thus decreasing the phosphorylation of RLC actually incorporated in myosin molecules.} \]

**Effects of Myosin RLC Phosphorylation in Cell Division**—The bulk of evidence has indicated that myosin plays a critical role in cell division. It is less clear, however, whether or not myosin RLC phosphorylation to activate myosin motor activity is critical for cell division in mammalian cells. We addressed this question by overexpressing unphosphorylatable RLC in mitotic mammalian cells. It has been known that myosin II accumulates at the contractile ring during cell division (12–16, 31). We first examined whether or not myosin containing RLC-GFP localizes at the contractile ring during mitosis. As shown in Fig. 5, A (early telophase) and C (late telophase), the GFP signal of the wild type RLC-GFP-transfected cells was concentrated at the contractile ring, suggesting that RLC-GFP containing myosin shows authentic localization during mitosis. Immunostaining of COS-7 cells with anti-phospho-RLC antibody during cytokinesis showed staining at the equator of dividing cells, indicating that myosin in the contractile ring is phosphorylated at its RLC. Therefore, we anticipated that the introduction of unphosphorylatable RLC into myosin in the cells would decrease the fraction of phosphorylated myosin and thus hamper proper mitosis. Wild type RLC-GFP or T18A/S19A RLC-GFP was transfected into COS-7 cells, and the number of nuclei per cell was observed at 72 h after transfection. A number of cells transfected by T18A/S19A RLC-GFP became multinuclear cells. Fig. 6 shows such a transfected cell. Cells transfected by both wild type and mutant RLC-GFP showed filamentous localization of GFP fluorescence (Fig. 6a, panels 1 and 2), indicating that both chimeric RLCs are incorporated into myosin molecules and confer proper myosin localization. However, mutant RLC-GFP-transfected COS-7 cells became multinuclear cells (Fig. 6a, panel 4) compared with wild type RLC-GFP-transfected cells (Fig. 6a, panel 3) and GFP-transfected control cells (data not shown). Approximately 40% of the mutant RLC-GFP-transfected cells had multiple nuclei. At 72 h after transfection, the fraction of multinuclear cells was 2.5-fold greater for mutant RLC-GFP-transfected cells than for wild type RLC-GFP-transfected cells (Fig. 6b). Although GFP-tagged RLC demonstrated normal myosin function using various criteria, i.e. normal binding to the heavy chain, normal distribution of myosin in cells, and normal response to ConA signal during the capping process, it is possible that the GFP tag may induce abnormal cell division. To address this possibility, we subcloned RLC cDNA into a pTracer-CMV mammalian expression vector and transfected cells with this construct. The pTracer-CMV expression vector contains a super GFP expression cassette under the control of an SV40 promoter, with the inserted foreign gene under the control of a cytomegalovirus promoter. Therefore, transfected cells expressing non-GFP-tagged RLC can be selected by the fluorescence of the independently expressed GFP. Transfection of COS-7 cells with untagged RLCs

**TABLE I**

**ATPase activities of RLC-GFP myosin II**

The activities were measured with three independent experiments. All data are presented as mean activity ± S.E.

|          | Dephosphorylated | Phosphorylated |
|----------|------------------|----------------|
|          | −Actin +Actin    | −Actin +Actin  |
| Wt RLC-GFP | 0.86 ± 0.09      | 3.49 ± 0.16    |
| T18A/S19A RLC-GFP | 1.67 ± 0.03 | 20.45 ± 2.8    |

* Values are shown in nmol/min · mg.
showed the same effects in ConA-induced capping formation as those obtained with the overexpression of GFP-tagged RLCs, i.e., T18A/S19A RLC overexpression inhibited cap formation (data not shown). As shown in Fig. 7A, transfection of COS-7 cells with non-tagged T18A/S19A RLC significantly increased (5.5-fold) the fraction of multinuclear cells compared with wild type RLC expressing cells. In NRK cells, the fraction of multinuclear cells was 8 times higher for cells transfected with mutant RLC versus wild type RLC (Fig. 7B). These results suggest that phosphorylation of myosin at Ser-19 and/or Thr-18 of RLC plays an important role in the normal cell division of mammalian somatic cells.

Disruption of RLC Phosphorylation of Myosin Causes Abnormal Cytokinesis and Chromosome Segregation—A remaining question is how the disruption of myosin phosphorylation at Myosin II Phosphorylation and Cytokinesis

Table II
Effect of RLC phosphorylation on ConA capping structure
Table represents the number of cells.

|              | Wt RLC-GFP | T18A/S19A RLC-GFP | Non-transfected |
|--------------|------------|-------------------|----------------|
| Capping (+)  | 267 (76.7 ± 1.1%) | 57 (27.8 ± 0.8%) | 526 (85.5 ± 2.1%) |
| Capping (-)  | 84 (23.3 ± 1.1%) | 148 (72.2 ± 0.8%) | 94 (14.5 ± 2.1%) |

Fig. 4. An excess amount of RLC-GFP does not inhibit MLCK-induced myosin phosphorylation. Myosin II was phosphorylated by MLCK in the presence of various concentrations of isolated RLC-GFP. Wild type RLC-GFP (Wt, lanes 2–4) or (T18A,S19A RLC-GFP, AA, lanes 5–7) were added to the reaction mixture at the indicated times (mol of RLC-GFP/mol of myosin). Phosphorylated myosin was subjected to SDS-polyacrylamide gel electrophoresis followed by autoradiography.

Fig. 5. Localization of RLC-GFP containing myosin and phosphorylated myosin during cytokinesis. A and C and B and D show GFP localization and phosphorylated RLC (intrinsic) accumulation under the ConA cap (indicated by arrowheads in A), and anti-phospho-RLC antibody stains under the cap (indicated by arrowhead in C). Cap formation is inhibited (E) in T18A/S19A RLC-GFP expressing cell (D). Cells were treated with ConA (E) and stained with anti-phospho-RLC antibody (F). Indirect immunostaining of the cells revealed that phosphorylated RLC accumulated under the ConA cap. Bar, 10 μm.
Ser-19/Thr-18 of RLC, which is critical for the activation of myosin motor activity, hampers cell division. To determine the effects of the disruption of myosin phosphorylation during mitosis in the somatic cell cycle, we monitored either wild type RLC or T18A/S19A RLC expressing cells during cell division by video microscopy. For both COS-7 and NRK cells, wild type RLC-transfected cells had no detectable changes in cell division progression after release from nocodazole arrest (Fig. 8, A–E and F–J). The transit times between anaphase and cytokinesis were similar to those of untransfected cells (data not shown). A majority of both untransfected and wild type RLC-transfected cells exhibited the onset of anaphase within 40 min and completed cell division within 49 min after the release of mitotic arrest (Table III). In contrast, the T18A/S19A RLC expressing cells showed significantly delayed progression from metaphase to telophase (Table III), and many cells stayed at metaphase at 49 min after release (Fig. 9, A–C). The cells expressing T18A/S19A RLC showed a significantly hampered phenotype at cytokinesis, in which a distorted cleavage furrow was formed and normal cytokinesis failed (Fig. 9, D–H) (Table IV). Whereas the equatorial plane of the cell narrowed to certain extent, the arrangement of the contractile ring was hampered, and the contraction of the contractile ring was not completed to divide the cell. On the other hand, cells expressing the wild type RLC showed a normal arrangement of the contractile ring and proceeded through normal cytokinesis. These observations suggest that the inhibition of myosin phosphorylation at Thr-18/Ser-19 of RLC hampers both normal progression of mitosis and cytokinesis.

**DISCUSSION**

Actomyosin motor activity is thought to play a critical role in various aspects of cell motility such as cell locomotion, receptor
study, we have approached this question by overexpressing unphosphorylatable RLC in mammalian somatic cells. It is known that phosphorylation at Ser-19 and Thr-18 of RLC is mandatory for the motor activity of vertebrate non-muscle and smooth muscle myosins (see Refs. 22–24 for reviews). Previously, it was shown that substitution of these residues by Ala abolishes myosin motor activity (41). Furthermore, it was shown that this substitution also hampers the thick filament formation of myosin in vitro (41). Therefore, the effects of overexpression of T18A/S19A RLC in cell motile activity may be due to either diminished actomyosin contractile activity or diminished recruitment of myosin into filamentous structures. One way to obtain RLC overexpressing cells is to produce RLC overexpressing cell lines, but we avoided this approach in this study because of the following. 1) The cell phenotype may change during the establishment of a cell line due to secondary effects of overexpression. 2) The overexpression of unphosphorylatable RLC may cause the cell not to proliferate properly because of the disruption of mitosis. In order to identify transiently transfected cells, GFP fluorescence was utilized in the present study. Two approaches were taken. First, RLC was tagged with GFP. The advantage of this approach is that the localization of myosin during cell motility can be directly monitored. Therefore, we employed this approach in most of the experiments in this study. The GFP-tagged RLC showed normal myosin heavy chain binding activity in vitro. Furthermore, RLC-GFP showed stress fiber localization in interphase cells and a localization at the cleavage furrow in mitotic cells. Therefore, we concluded that myosin containing RLC-GFP retains the authentic properties of myosin. Nevertheless, to ensure our findings further, RLC cDNA was cloned into a pTracer vector containing a separate GFP expression cassette. The transfectants were injected into the vector expressing cells. Practically identical results were obtained using both approaches in both cell types for the motile events examined in this study, i.e. surface capping and cell division.

We first demonstrate that deletion of RLC phosphorylation sites by substitution of Ser-19/Thr-18 by Ala inhibits the induction of the receptor cap structure. The antibody that recognizes Ser-19 phosphorylation of RLC was detected under the ConA cap in both non-transfected and wild type RLC-GFP transfected cells but not T18A/S19A RLC-GFP-transfected cells. These results clearly indicate that the myosin RLC phosphorylation at Ser-19 is critical for receptor capping. Previously, it was reported that overall RLC phosphorylation in whole cell extracts increases during the capping event (9) and that actomyosin-containing structures are associated with cap formation (5–11). The present results are consistent with these earlier findings and provide direct evidence that myosin phosphorylation is required for receptor capping in mammalian cells. It was reported previously that, in Dictyostelium, ConA-induced capping was unaffected by disruption of a calmodulin-independent MLCK (mlck-A) gene (47), even though mlck-A increases RLC phosphorylation of Dictyostelium myosin by treatment with ConA. The discrepancy from the present results is likely to be due to a difference in the regulation between the two myosins. Whereas RLC phosphorylation is required for myosin motor activity in vertebrate smooth muscle and non-muscle myosin, RLC phosphorylation does not significantly augment the actin translocating activity of Dictyostelium myosin (48).

It has been assumed that the activation of myosin contractile activity is important for mitosis, especially during cytokinesis, since actomyosin is enriched at the cleavage furrow and forms

### Table III

| Effect of the disruption of RLC phosphorylation on cell division |
|---------------------------------------------------------------|
| COS-7 cells were transfected with pTracer-CMV vector containing |
| either wild type RLC cDNA or T18A/S19A RLC cDNA. 72 h after  |
| transfection, the cells were fixed at indicated times after the  |
| release of nocodazole arrest and stained with anti-GFP antibody  |
| and DAPI. More than 300 individual cells were counted from three |
| independently transfected cells. Results represent the mean ± S.E. |

|                            | Wild type RLC | T18A/S19A RLC |
|-----------------------------|---------------|---------------|
| Metaphase                   | 90.6 ± 4.1    | 90.7 ± 2.2    |
| Anaphase onset to telophase | 9.4 ± 4.1     | 9.3 ± 2.2     |
| Cytokinesis                 | 0 ± 3.0       | 32.2 ± 3.7    |

### Table IV

| Formation of abnormal cleavage furrow |
|---------------------------------------|
| COS-7 cells were transfected with pTracer-CMV vector containing either wild type RLC or T18A/S19A RLC cDNA. 72 h after transfection, the cells were fixed at 55 min after the release of nocodazole arrest and stained with anti-GFP antibody and DAPI. Results represent the mean ± S.E. of three independent experiments. |

| Cleavage furrow | Normal | Distortion |
|-----------------|--------|------------|
| Wild type RLC (n = 122) | 100% | 0% |
| T(18,19A) RLC (n = 110) | 85.1 ± 1.9% | 14.9 ± 1.0% |

capping, and cell division. In vertebrate and non-muscle cells, myosin motor activity in vitro is regulated by phosphorylation of RLC at Ser-19 and Thr-18, especially at the former site. Therefore, the cell motile processes that are governed by actomyosin contractile activity are assumed to be controlled by a dynamic change in RLC phosphorylation during these processes. Yet the relationship between myosin phosphorylation and these cell movements is not well understood. In the present...
the contractile ring that narrows during cell division (12–15). Although it is accepted that actomyosin function is crucial for cell division, it is unclear how actomyosin contractile activity is regulated during mitosis in vertebrate cells. As vertebrate myosin motor activity is regulated by RLC phosphorylation, it is reasonable to assume that actomyosin function and thus myosin phosphorylation is temporally and spatially regulated during mitosis, but the functional significance of serine 19 phosphorylation of myosin II RLC during cell division in mammalian cells is not understood. It has been proposed that phosphorylation of myosin at Ser-19 of RLC takes place in the equatorial contractile ring and activates contraction of the cleavage furrow after the dephosphorylation of RLC at the Cdc2 kinase sites (49), but the significance of the phosphorylation at the Cdc2 kinase sites during cell division is questioned (50). The present results show that the cells expressing the unphosphorylatable form of RLC become multinucleated, suggesting an abnormal cell dividing process, which is consistent with this model. Microscopic observation of mitotic cells revealed that there are two types of defects in the cells expressing unphosphorylatable RLC. One is the formation of an abnormal cleavage furrow followed by the failure to complete proper cytokinesis. The other is the inhibition of transition from metaphase to anaphase. As shown in Fig. 9, T18A/S19A RLC expressing cells showed a distorted cleavage furrow. This result suggests that the contractile ring is not properly arranged at the equator of the cells. Previously, Wang and co-workers (34) observed that NRK cells microinjected with constitutively active MLCK had a delayed onset of anaphase, but the treatment did not alter the rate of progression of cytokinesis. It has also been demonstrated (51) that the level of RLC phosphorylation at Ser-19/Thr-18 increases at the cell stage just prior to entering cytokinesis, and the phosphorylation level is maintained during cell division. Therefore, it would be plausible that myosin II phosphorylation is elevated before myosin is recruited into the contractile ring, and the disruption of myosin phosphorylation may alter the formation of a normal contractile ring.

An interesting finding is that the overexpression of unphosphorylatable RLC disrupted the movement of chromosomes from the metaphase plate, suggesting that myosin plays an important role in the progression from metaphase to anaphase and that myosin phosphorylation is involved in properly performed karyokinesis. This view is supported by the previous finding that microinjection of constitutively active MLCK delayed the onset of anaphase (34). These results suggest that proper myosin phosphorylation during mitosis is critical for normal transition from metaphase to anaphase. Although the mechanism by which activation of myosin function influences chromosome separation is unclear, it was reported that anti-myosin IIA antibody stains the spindle poles between prophase and metaphase (31). Recently, it was reported (16) that anti-phospho-RLC polyclonal antibody stained the spindle poles, although since this antibody also recognized other proteins it remained uncertain whether or not phospho-RLC was present there. However, our monoclonal anti-phospho-RLC antibody also recognized the spindle poles during transition from prophase to metaphase, supporting the presence of phosphorylated myosin. The present findings, together with these observations, suggest that myosin phosphorylation is involved not only in cytokinesis but also in the separation of sister chromatids. It was previously found that microinjection of antimyosin II antibodies into starfish eggs inhibited cytokinesis (32), but this also produced an abnormal mitotic apparatus. These results suggest that anti-myosin II antibodies might influence the mitotic apparatus. The expression of T20A/S21A RLC in Drosophila RLC null germ line cells showed an increase in multinucleated cells (52), resembling the results of the present study. The Drosophila experiment detected abnormal cytokinesis, but an abnormality of karyokinesis was not reported. The difference from the present study could be due to the following: 1) a difference between mitosis during oogenesis and during the somatic cell cycle (present study); 2) a difference in the regulation of mitosis between Drosophila and mammalian cells; and 3) a difference between the regulation of myosin II in mammalian cells and in Drosophila (which is not well understood at a molecular level). The myosin RLC null mutant of Dicyostelium showed a deficiency in cytokinesis and produced multinucleated cells (48, 53). However, the effect on cytokinesis is not well studied. It should be noted that RLC null cells retain myosin ATPase activity (1/3 of the wild type) and that RLC domain-deficient myosin of Dicyostelium showed more than 50% of the motility activity of the wild type myosin. These results suggest that Dicyostelium RLC null myosin retains some motor activity. Therefore, it is plausible that the inhibition of each biological process related to myosin II activity depends on the extent of the inhibition of myosin II function. Alternatively, the involvement of myosin II in karyokinesis might be developed in mammalian cells but not in lower eukaryotes. Recently, Simerly et al. (54) reported that the injection of myosin IIA antibody disrupts the eccentric positioning of the metaphase spindle during meiotic maturation, probably through depletion of spindle-associated myosin IIA. The present result together with earlier results suggest the involvement of myosin II function in proper chromosomal segregation during mitosis.

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REFERENCES
1. Wilson, A. K., Gorgas, G., Claypool, W. D., and de Lanerolle, P. (1991) J. Cell Biol. 114, 277–283
2. Valerius, N. H., Stendahl, O., Hartwig, J. H., and Stossel, T. P. (1981) Cell 24, 195–205
3. Zigmund, S. H., and Hirsch, J. G. (1972) Exp. Cell Res. 73, 383–393
4. Korn, E. D. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 588–599
5. Schreiner, G., Fujikawa, K., Pollard, T. D., and Un Ones, E. (1977) J. Exp. Med. 145, 1395–1398
6. Bourguignon, L. Y. W., and Singer, S. J. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5031–5035
7. Bourguignon, L. Y. W., Tokuyasu, K. T., and Singer, S. J. (1978) J. Cell. Physiol. 96, 239–257
8. Bourguignon, L. Y. W. (1980) Cell Biol. Int. Rep. 4, 541–547
9. Bourguignon, L. Y., Nagpal, M. L., and Hsing, Y. C. (1981) J. Cell Biol. 91, 889–894
10. Flanagan, J., and Koch, G. L. E. (1978) Nature 273, 278–281
11. Condeelis, J. (1979) J. Cell Biol. 80, 751–758
12. Fujikawa, K., and Pollard, T. D. (1976) J. Cell Biol. 71, 848–875
13. Aubin, J. E. K., Weber, and Osborn, M. (1979) Exp. Cell Res. 124, 93–109
14. Maupin, P., and Pollard, T. D. (1986) J. Ultrastruct. Mol. Struct. Res. 94, 92–103
15. Sanger, J. M., Mittal, B., Dome, J. S., and Sanger, J. W. (1989) Mol. Cell. Cytoskeleton 14, 201–219
16. Matsumura, F., Ono, S., Yamakita, Y., Totsukawa, G., and Yamashiro, S. (1998) J. Cell Sci. 111, 119–129
17. Mabuchi, I. (1986) Int. Rev. Cytol. 101, 175–213
18. Salmon, E. D. (1989) Curr. Opin. Cell Biol. 1, 541–547
19. Sellers, J. R. (1991) Curr. Opin. Cell Biol. 3, 98–104
20. Trybus, K. M. (1991) Cell Motil. Cytoskeleton 18, 81–85
21. Tan, J. L., Ravid, S., and Spudich, J. A. (1992) Annu. Rev. Biochem. 61, 721–759
22. Hartshorne, D. J. (1987) in Physiology of the Gastrointestinal Tract (Johnson, L. R., ed) 2nd Ed., pp. 423–482, Raven Press, Ltd., New York
23. Sellers, J. R., and Adelstein, R. S. (1987) in The Enzymes, Vol. 18, pp. 381–418, Academic Press, New York
24. Kamm, K. E., and Stull, J. T. (1989) Annu. Rev. Physiol. 51, 299–313
25. Edelman, A. M., Lin, W. H., Osterhout, D. J., Beneit, M. K., Kennedy, M. B., and Krebs, E. G. (1989) Mol. Cell. Biochem. 97, 87–98
26. Amamoto, M., Ho, K., Kinura, Y., Fukata, R., Chihiro, T., Nakano, Y., Matsunaka, Y., and Kibuchi, K. (1996) J. Biol. Chem. 271, 20246–20249
27. Komatsu, S., and Hosoya, H. (1996) Biochem. Cell Biol. 223, 741–745
28. Nishikawa, M., Sellers, J. R., Adelstein, R. S., and Hidaka, H. (1984) J. Biol.
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Chen, 259, 8808–8814
29. Bengur, A. R., Robinson, E. A., Appella, E., and Sellers, J. R. (1987) J. Biol. Chem. 262, 7613–7617
30. Ikebe, M., Hartshorne, D. J., and Elzinga, M. (1987) J. Biol. Chem. 15, 9569–9573
31. Kelley, C. A., Sellers, J. R., Gard, D. L., Bui, D., Adelstein, R. S., and Baines, I. C. (1996) J. Cell Biol. 134, 675–687
32. Mabuchi, I., and Okuno, M. (1977) J. Cell Biol. 74, 251–263
33. De Lozanne, A., and Spudich, J. A. (1987) Science 236, 1086–1091
34. Fishkind, D. J., Cao, L. G., and Wang, Y. L. (1991) J. Cell Biol. 114, 967–975
35. Ikebe, M., and Hartshorne, D. J. (1985) J. Biol. Chem. 260, 19569–19573
36. Spudich, J. A., and Watt, S. (1971) J. Biol. Chem. 246, 4866–4871
37. Chien, Y. H., and Davids, J. B. (1984) Mol. Cell. Biol. 4, 507–513
38. Ikebe, M., Reardon, S., Schone, J. P., Sanders, C. R., II, and Ikebe, R. (1994) J. Biol. Chem. 269, 25165–25172
39. Ikebe, M., Kambara, T., Stafford, W. F., Sata, M., Katayama, E., and Ikebe, R. (1998) J. Biol. Chem. 273, 17792–17797
40. Yano, T., Taura, C., Shibata, M., Hirano, Y., Ando, S., Kusuhara, M., Takahashi, T., and Inagaki, M. (1991) Biochem. Cell Biol. 175, 1144–1151
41. Kamisoyama, H., Araki, Y., and Ikebe, M. (1994) Biochemistry 33, 840–847
42. Yano, K., Araki, Y., Hales, S. J., Tanaka, M., and Ikebe, M. (1993) Biochemistry 32, 12054–12061
43. Lammli, U. K. (1970) Nature 227, 680–685
44. Higashihara, M., Prado, L. L., Craig, R., and Ikebe, M. (1989) J. Biol. Chem. 264, 5218–5225
45. Perrie, W. T., and Perry, S. V. (1970) Biochem. J. 119, 31–38
46. Carrington, W. A., Lynch, R. M., Moore, E. D., Isenberg, G., Fogarty, K. E., and Fay, F. S. (1993) Science 268, 1483–1487
47. Smith, J. L., Silveira, L. A., and Spudich, J. A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 12321–12326
48. Ostrow, B. D., Chen, P., and Chisholm, R. L. (1994) J. Cell Biol. 127, 1945–1955
49. Pollard, T. D., Satterwhite, L., Cusek, L., Corden, J., Sato, M., and Maupin, P. (1990) Ann. N. Y. Acad. Sci. 582, 120–130
50. Shuster, C. B., and Burgess, D. R. (1999) J. Cell Biol. 146, 981–992
51. Yamakita, Y., Yamashiro, S., and Matsumura, F. (1994) J. Cell Biol. 124, 129–137
52. Jordan, P., and Karess, R. (1997) J. Cell Biol. 139, 1805–1819
53. Chen, P., Ostrow, B. D., Tafuri, S. R., and Chisholm, R. L. (1994) J. Cell Biol. 127, 1933–1944
54. Simerly, C., Nowak, G., de Lanerolle, P., and Schatten, G. (1998) Mol. Biol. Cell 9, 2509–2525