

# Mechanism of the Formation of Contractile Ring in Dividing Cultured Animal Cells.

## II. Cortical Movement of Microinjected Actin Filaments

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**Abstract.** The contractile ring in dividing animal cells is formed primarily through the reorganization of existing actin filaments (Cao, L.-G., and Y.-L. Wang, 1990. *J. Cell Biol.* 110:1089–1096), but it is not clear whether the process involves a random recruitment of diffusible actin filaments from the cytoplasm, or a directional movement of cortically associated filaments toward the equator. We have studied this question by observing the distribution of actin filaments that have been labeled with fluorescent phalloidin and microinjected into dividing normal rat kidney (NRK) cells. The labeled filaments are present primarily in the cytoplasm during prometaphase and early metaphase, but become associated extensively with the cell cortex 10–15 min before the onset of anaphase. This process is manifested both as an increase in cortical fluores-

cence intensity and as movements of discrete aggregates of actin filaments toward the cortex. The concentration of actin fluorescence in the equatorial region, accompanied by a decrease of fluorescence in polar regions, is detected 2–3 min after the onset of anaphase. By directly tracing the distribution of aggregates of labeled actin filaments, we are able to detect, during anaphase and telophase, movements of cortical actin filaments toward the equator at an average rate of 1.0  $\mu\text{m}/\text{min}$ . Our results, combined with previous observations, suggest that the organization of actin filaments during cytokinesis probably involves an association of cytoplasmic filaments with the cortex, a movement of cortical filaments toward the cleavage furrow, and a dissociation of filaments from the equatorial cortex.

PAST studies have indicated critical roles of actin and myosin in the process of cytokinesis (for reviews see Mabuchi, 1986; Salmon, 1989). Of particular interest is the structure of the contractile ring on the equatorial plane of dividing cells (Schroeder, 1970). It contains a relatively high concentration of both actin and myosin II filaments (Perry et al., 1971; Fujiwara and Pollard, 1976) and is considered to be responsible for the generation of contractile forces for cytokinesis.

The contractile ring is known to be a transient structure. Its assembly can be detected during late anaphase as an increase in the concentration of actin and myosin on the equatorial plane. The onset of disassembly is uncertain but appears to occur well before the completion of cytokinesis, as suggested by the constant thickness of the contractile ring despite the decrease of its diameter (Schroeder, 1972).

Various possibilities have been raised regarding the mechanism of the assembly of the contractile ring and the signals involved (discussed recently in Harris and Gewalt, 1989; Devore et al., 1989). In the first report of this series (Cao and Wang, 1990), we have described the microinjection of fluorescent phalloidin and fluorescent actin monomers to probe the redistribution of existing filaments and the sites of de novo filament assembly, respectively. Although some in-

corporation of actin subunits appears to occur on the equatorial plane, the results are more consistent with the reorganization of existing filaments being the primary mechanism for the formation of the contractile ring. Much less clear, however, is how this reorganization takes place. Two possibilities may account for the concentration of filaments into the cleavage furrow. First, actin filaments in the contractile ring may be recruited from the cytoplasmic pool, possibly as a result of an increase in the affinity or the number of binding sites for actin filaments in the equatorial cortex (Schroeder and Otto, 1988). In the second mechanism, membrane-associated filaments may move along the cell cortex from the poles toward the equator (White and Borisy, 1983). The convergence of flow from the opposite hemispheres of the dividing cell then gives rise to the increase in the concentration of actin and myosin.

The purpose of the present study is to determine how actin filaments become concentrated in the cleavage furrow, by directly following the distribution of exogenous actin filaments that have been labeled with rhodamine phalloidin (rh-pha)<sup>1</sup> and microinjected into dividing normal rat kidney

1. *Abbreviations used in this paper:* NRK, normal rat kidney; rh-pha, rhodamine phalloidin.

(NRK) cells. Our results suggest that two events probably occur during the formation of the contractile ring. The first is an increase in the association of actin filaments over the entire cortex during late metaphase. This is then followed by a directional movement of cortical filaments toward the equatorial plane during anaphase and telophase.

## Materials and Methods

### Preparation of Actin, Fluorescent Phalloidin, and Beads

rh-pha was purchased from Molecular Probes Inc. (Eugene, OR) as a 3.3  $\mu\text{M}$  stock solution in methanol. In a typical experiment, 400  $\mu\text{l}$  of the stock solution was dried under  $\text{N}_2$  and redissolved in 40  $\mu\text{l}$  of 5 mM Tris-acetate, pH 6.95. The solution was clarified at 25,000 rpm for 20 min in a type 42.2 Ti rotor (Beckman Instruments, Inc., Palo Alto, CA).

Muscle actin was purified from rabbit back and leg muscles according to the method of Spudich and Watt (1971). G-actin was dialyzed overnight in a buffer of 2 mM Tris-acetate, 0.05 mM  $\text{MgCl}_2$ , 0.2 mM ATP, and 0.2 mM DTT, pH 6.95, and clarified at 25,000 rpm for 20 min in a type 42.2 Ti rotor. Polymerization was induced by adding  $\text{MgCl}_2$  to 2 mM. The F-actin was then mixed with rh-pha to obtain a rh-pha/actin molar ratio of 1.0 to 1.2 and a final actin concentration of 4.2  $\mu\text{M}$ . Before microinjection, actin filaments were sheared by sonication for 5–10 s in a bath sonicator or by pipetting up and down 30–40 times through a gel-loading pipette tip.

Uncharged fluorescent polystyrene beads were purchased from Polysciences, Inc. (Warrington, PA; 0.1  $\mu\text{m}$  plain YG Fluoresbrite microspheres). The beads were centrifuged and resuspended in 10 mg/ml BSA (Sigma Chemical Co., St. Louis, MO), 5 mM Tris-acetate, pH 6.95. Microinjection was performed after sonication in a bath sonicator.

### Cell Culture and Microinjection

NRK epithelial cells (NRK-52E; American Type Culture Collection, Rockville, MD) were cultured in the F12K medium (Hazleton Systems, Inc., Lenexa, KS) containing 10% Nu-serum (Collaborative Research, Bedford, MA), 50  $\mu\text{g}/\text{ml}$  streptomycin, and 50 U/ml penicillin, and were maintained at 36–37°C under 5%  $\text{CO}_2$ . Cells were plated onto special coverslip dishes and maintained on the microscope stage as described previously (McKenna and Wang, 1989) at a temperature of 32–33°C.

Cells at various stages of mitosis were identified and microinjected as described in Cao and Wang (1990). The solutions were microinjected conservatively, and the volume delivered is estimated to be <5% of the cell volume. No cell damage was visible under the phase optics, and microinjected cells divided normally, following a similar time course as did neighboring uninjected cells.

### Staining of Cells with Fluorescent Phalloidin or with Hoechst 33258

Cells were rinsed twice with warm PHEM buffer (Schliwa and van Blerkom, 1981; 10 mM EGTA, 2 mM  $\text{MgCl}_2$ , 60 mM Pipes, and 25 mM Hepes, pH 6.9), fixed for 10 min with 3.2% formaldehyde (from 16% stock, EM Sciences, EM Industries, Inc., Cherry Hill, NJ) in PHEM buffer, pH 6.1, then extracted for 5 min with acetone chilled in dry ice. The coverslip was rinsed twice with PHEM buffer and stained with fluorescein phalloidin (Molecular Probes Inc.) for 10 min in PHEM buffer at a concentration of 220 nM.

Chromosomes were stained by replacing medium with culture medium containing Hoechst 3358 (Sigma Chemical Co.) at a concentration of 10  $\mu\text{g}/\text{ml}$ . After incubation for 40 min, the dye-containing medium was replaced with fresh medium and the cells were incubated for 1 h before microinjection.

### Fluorescence Microscopy and Image Processing

Fluorescence microscopy was performed as described previously (Cao and Wang, 1990). Image acquisition and processing were performed with a model 3120 Workstation (Silicon Graphics, Mountain View, CA) in conjunction with series No. 150 image processing boards (Imaging Technology, Inc., Woburn, MA). For the detection of extremely faint structures, 300 video frames were summed into a 16-bit frame buffer, and the background

obtained by summing 300 dark images was subtracted. The resulting images were divided by two repeatedly until the maximal pixel value became <256 (and thus can be displayed as 8-bit images). Time-lapse sequences were recorded at a 30–60-s interval. Moving structures were identified by displaying images in rapid succession with the image processor (Wang, 1990) and were analyzed as in McKenna et al. (1989).

## Results

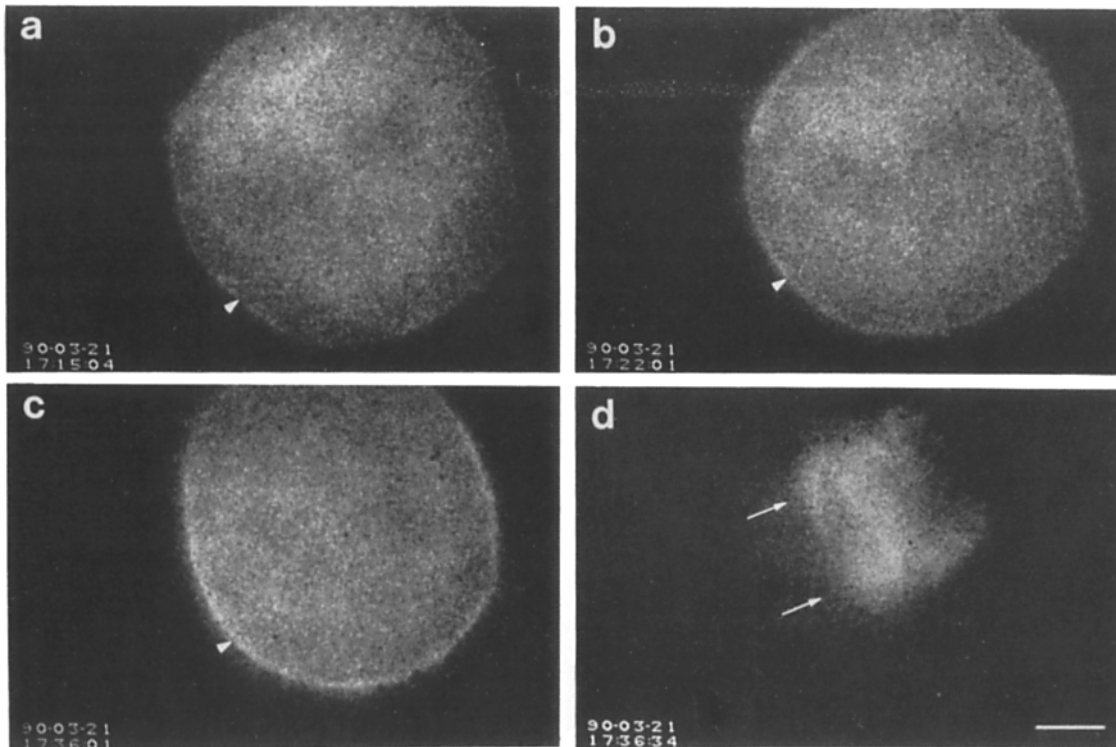
### Distribution of Microinjected Actin Filaments in Dividing Cells

We have microinjected a trace amount of F-actin labeled with rh-pha into dividing NRK cells in order to follow the distribution of actin filaments during cell division. The injected actin was initially localized near the site of microinjection but dispersed within 10–30 min. The rate of dispersal in mitotic cells was much higher than that in interphase cells (Sanders and Wang, 1990) and varied according to the degree that actin filaments had been sheared. Fluorescence associated with well-sonicated fragments dispersed from the site of microinjection within 10 min, without breaking down into smaller aggregates. However, gently sheared filaments often formed multiple aggregates after microinjection, which remained detectable for an extended period of time (discussed later). In neither case did the microinjection induce detectable changes in cellular morphology or affect the time course of cell division.

The distribution of actin filaments was first studied with well-sonicated fragments microinjected into prometaphase cells. After dispersion from the site of microinjection, the fluorescence remained diffuse within the cytoplasm during prometaphase and early metaphase (Fig. 1 *a*). In some cells a very slight concentration in the cortex was observed (Fig. 1 *a*). The cortex showed an increase in intensity at late metaphase (Fig. 1 *b*), 10–15 ( $11 \pm 2.5$  SEM,  $n = 13$ ) min before the onset of anaphase, and became readily detectable at the onset of anaphase (Fig. 1 *c*; chromosomal distribution shown in Fig. 1 *d*). During anaphase, the fluorescent actin became relocated gradually onto the equatorial plane, such that a clear concentration of fluorescence in the furrow could be observed 2–3 min after the onset of anaphase (Fig. 2). However, since no discrete structures could be resolved, it was difficult to determine whether or not the reorganization involves a directional movement of actin filaments. The fluorescence intensity in the polar regions showed a concomitant decrease in all cells that rounded up during cytokinesis (Fig. 2 *b*). However, the decrease was less pronounced in a small fraction of cells that maintained numerous processes and remained spread during cytokinesis.

### Cortical Movement of F-actin

Gently sheared actin filaments, which remained as visible aggregates during cytokinesis, were microinjected at anaphase to allow direct observations of the movement of actin filaments. The site of injection was located outside the mitotic spindle, approximately halfway between the equator and one of the poles. The size and number of aggregates varied according to the extent that actin had been sheared. Large aggregates of actin usually stayed near the site of microinjection and underwent a gradual decrease in intensity without a clear movement. However, smaller aggregates were ob-

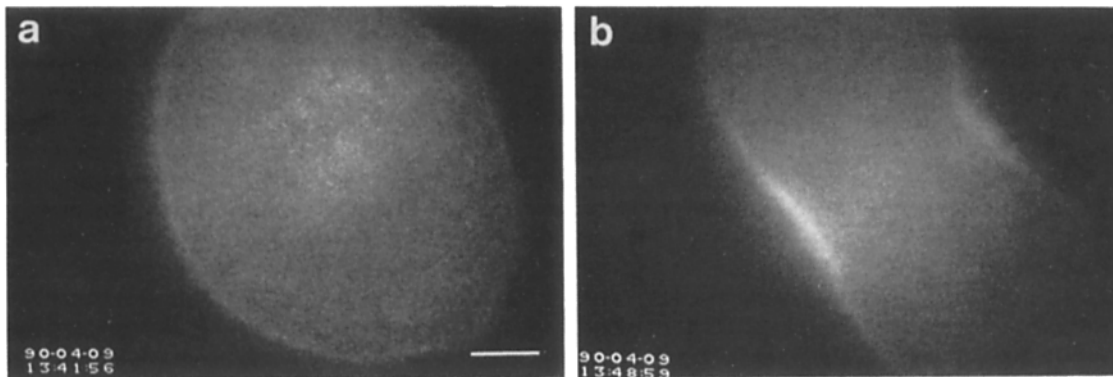


**Figure 1.** Distribution of fluorescently labeled actin filaments in dividing NRK cells during metaphase and early anaphase. The cell was labeled with Hoechst 33258 and microinjected with sonicated actin filaments during prometaphase. rh-pha-labeled actin filaments are distributed more or less uniformly in the cytoplasm during metaphase (*a*). Limited association with the cortex can be observed in some areas (*a*, *arrowhead*). The association with the cortex starts to increase  $\sim 13$  min before anaphase (*b*, *arrowhead*), and becomes readily detectable at the onset of anaphase (*c*, *arrowhead*; taken 1 min after the onset of anaphase). The two groups of separating chromosomes, stained by Hoechst 33258, were recorded  $\sim 2$  min after the onset of anaphase using a different filter set (*d*, *arrows*). Bar, 5  $\mu\text{m}$ .

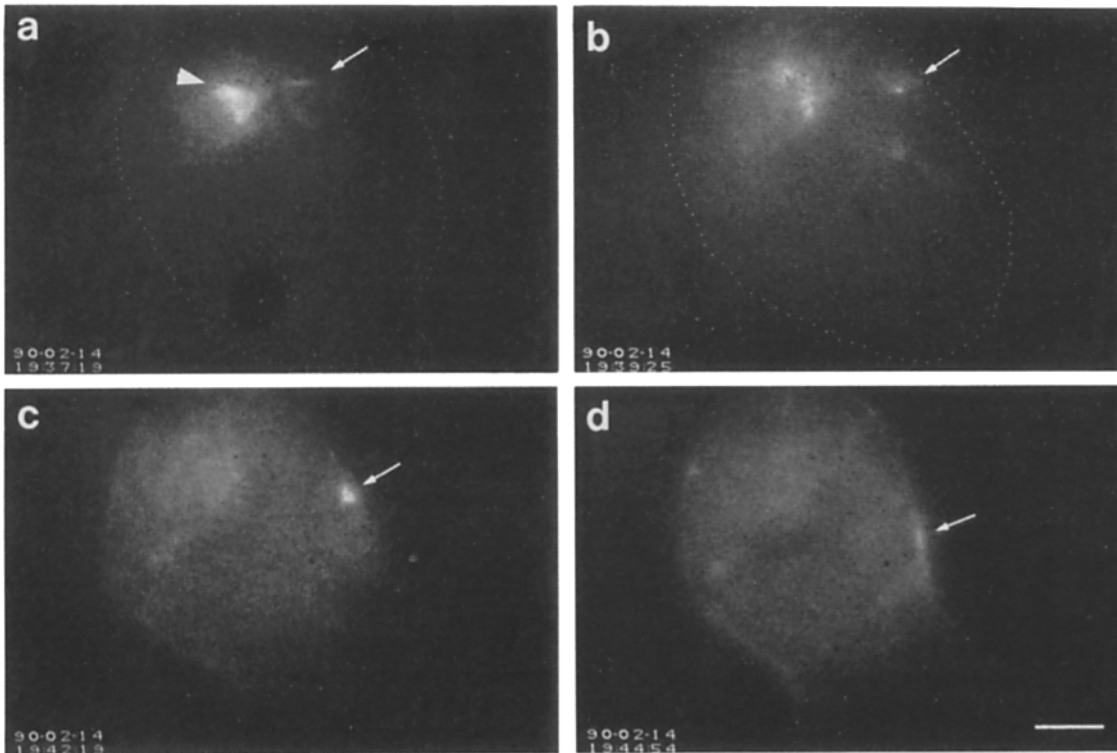
served to separate from the site of microinjection and in some cases move toward the edge of the cell and became associated with the cortex (Fig. 3). Like well-sonicated filaments, gently sheared actin filaments became concentrated on the equatorial plane during cytokinesis (Figs. 3–5), although the extent of concentration and the pattern of distribution were affected by the degree of dispersion from the site of microinjection. In cells injected during late anaphase, the

fluorescence was often concentrated on one side of the equatorial plane, near the site of microinjection (Fig. 5).

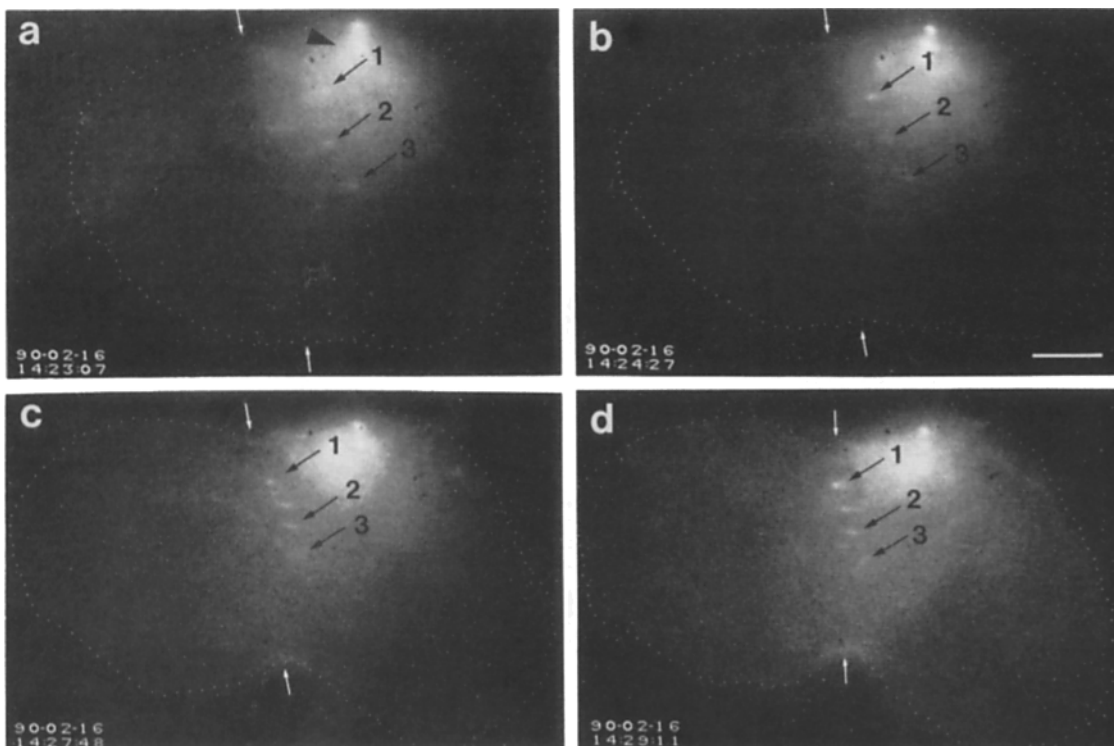
Directional movement of actin toward the cleavage furrow was observed with small aggregates located both along the edge (Fig. 3) and on the top surface (Fig. 4) of the cell. The movement showed an average rate of 1.0  $\mu\text{m}/\text{min}$  relative to the substrate ( $\pm 0.2$  SEM,  $n = 10$ ) and proceeded without a detectable reversal in direction until the aggregates reached



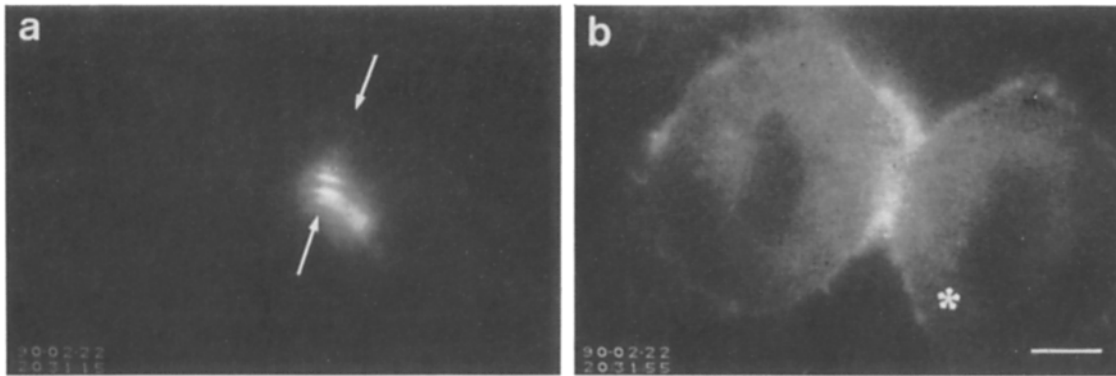
**Figure 2.** Redistribution of fluorescently labeled actin filaments into the cleavage furrow during cytokinesis. A cell was microinjected with sonicated actin filaments during prometaphase as in Fig. 1. The fluorescence is concentrated along the entire cortex  $\sim 2$  min before the onset of anaphase (*a*), but becomes redistributed into the cleavage furrow during cytokinesis (*b*). Intensities near polar regions show a concomitant decrease. Bar, 5  $\mu\text{m}$ .



**Figure 3.** Movement of actin filaments during anaphase and telophase. An NRK cell was microinjected during early anaphase with gently sheared, rh-pha-labeled actin filaments. A small aggregate (arrows) dissociates from the site of injection (a, arrowhead) 2-3 min after microinjection (a), and subsequently moves toward the edge of the cell (b, dotted line). It then becomes associated with the cortex during the early stage of cytokinesis (c), and moves into the cleavage furrow along the edge of the cell (d). Aggregates near the site of injection disappear over the period of observation. Boundaries of the cell during anaphase (dotted lines) were determined after extensive contrast enhancement. In both this sequence and Fig. 4, the culture dish remained stationary throughout the period of observation, and the time-date on the micrographs can be used as reference points. Bar, 5  $\mu$ m.



**Figure 4.** Movement of actin aggregates during telophase. The cell was microinjected as in Fig. 3. a was recorded 2-3 min after microinjection. The microscope was focused on the top surface of the cell, thus the edge of the cell (dotted lines) is not as clearly defined as in Fig. 3. Several aggregates, identified by numbers and black arrows, become separated from the site of microinjection and move into the cleavage furrow as cytokinesis proceeds. A large aggregate remains near the site of microinjection (a, arrowhead) throughout the period of observation. Equators are defined by white arrows. Bar, 5  $\mu$ m.



**Figure 5.** Longitudinal bundles of actin in the cleavage furrow. The cell was microinjected with pipette-sheared, rh-pha-labeled actin filaments during late anaphase, and was fixed and stained with fluorescein phalloidin during mid-cytokinesis. The image of rh-pha (*a*) shows several bundle-like structures lying almost perpendicular to the equator (delineated by white arrows). The bundle-like structures are concentrated near the lower side of the cell, where the microinjection was performed (approximate site marked by  $\star$  in *b*). The image of fluorescein phalloidin (*b*) shows a distribution typical of cells at this stage, with intense staining in the cleavage furrow but no visible longitudinal bundles. Bar, 5  $\mu\text{m}$ .

the cleavage furrow. Frequently the actin filaments merged into bundle-like structures perpendicular to the equator (Fig. 5). However, the pattern of the distribution of total actin filaments in such cells, as revealed by fixation and staining with fluorescein phalloidin, was identical to that of uninjected cells, and no bundle structures perpendicular to the equatorial plane could be resolved (Fig. 5 *b*). Thus the bundles probably represent labeled filaments aligned along defined paths as they moved into the cleavage furrow rather than artifactual structures nucleated by the injected filaments.

The movement of actin filaments was compared to that of fluorescent polystyrene beads microinjected into late metaphase cells. The beads showed no directional movement when microinjected away from the mitotic apparatus and exhibited poleward movement when microinjected near the mitotic apparatus. Similar poleward movement was reported in Echinoderm eggs by Hamaguchi et al. (1986) and by Wadsworth (1987). The behavior of the beads was clearly different from that of microinjected actin filaments, indicating that the as-

sociation with the cortex and the movement toward the equator may represent processes specific for actin filaments or actin-associated structures.

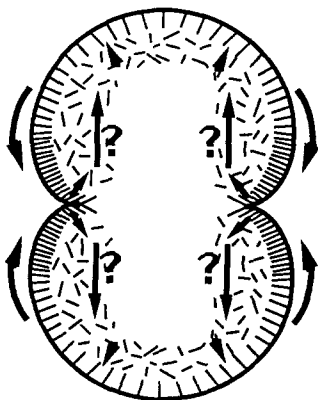
## Discussion

Because of the dynamic nature of the contractile ring, it has been very difficult to study the mechanism of its assembly and disassembly with conventional biochemical, immunofluorescence, or ultrastructural techniques. The microinjection of fluorescent analogues provides us with a powerful means for observing directly the distribution of specific components in living cells. In the present study we ask the questions, Do actin filaments undergo any directional movement during cell division, and how might the movement be related to the formation of the contractile ring?

We have microinjected, into dividing cells, actin filaments that had been fluorescently labeled and stabilized with rh-pha. The behavior of such injected filaments during mitosis, such as the association with the cortex during late metaphase and the concentration into the cleavage furrow during cytokinesis, closely parallels that of endogenous actin reported previously (Sanger, 1975; Kitanishi-Yumura and Fukui, 1989). In addition, we have observed similar distributions of fluorescence in cells microinjected with a trace amount of rh-pha alone (Cao and Wang, 1990), which labels endogenous F-actin, and with rh-pha-saturated exogenous F-actin. Thus, it is likely that the results observed with exogenous actin reflect the normal mechanism for the reorganization of actin filaments during cell division.

Our results demonstrate that exogenous actin filaments can be incorporated into the cleavage furrow. This is consistent with our previous conclusion that the contractile ring is formed primarily through the reorganization of existing actin filaments, rather than polymerization of new filaments (Cao and Wang, 1990). The present results further suggest that the concentration of actin into the cleavage furrow is probably achieved through a directional movement along the cortex. Although recruitment of actin filaments into the cortex was

**Figure 6.** A hypothetical model showing the path of actin movement during cytokinesis. Cytoplasmic actin filaments become associated with the cortex (arrowheads) and subsequently move toward the cleavage furrow (curved arrows). The filaments then dissociate from the cortex and re-enter the cytoplasm (short arrows). Poleward movement of filaments in the cytoplasm (long arrows, question marks), possibly as a result of interactions with the cortex, may bring the filaments back to the polar cortex.



also detected, the incorporation appears to occur throughout the entire cortex and is unlikely to account for the concentration in the equatorial region.

The cortical movement of actin filaments is suggested not only by the direct observation of the translocation of actin aggregates (Figs. 3 and 4), but also by the pattern of distribution of actin filaments during anaphase and telophase (Figs. 2 and 5). First, the increase of actin in the cleavage furrow was accompanied by a decrease of prelabeled filaments at the poles (Fig. 2; Cao and Wang, 1990), consistent with a flow that originates in polar regions and directs towards the equator. Second, when actin was microinjected during anaphase and underwent a limited dispersion during cytokinesis, the fluorescence was still capable of concentrating in the cleavage furrow on the side that received the microinjection. This suggests that the reorganization probably involves a bulk translocation of actin filaments. Third, although other explanations are possible, the observations of bundle-like structures perpendicular to the equator are also consistent with a cortical flow of actin filaments toward the equator (Fig. 5). Such axially oriented filaments have been observed previously in flattened *Dictyostelium* with immunofluorescence and polarization optics (Kitanishi-Yumura and Fukui, 1989; Fukui, 1990), and in higher animal cells with electron microscopy (Maupin and Pollard, 1986). They are probably difficult to resolve with phalloidin staining (Fig. 5 b) because of the high density of filaments in the furrow region (Maupin and Pollard, 1986), but may become visible when the distribution of labeled actin filaments is limited to small areas (Fig. 5 a).

A cortical flow of actin during cytokinesis is also consistent with previous studies of cortical organelles and membrane receptors. Dan (1954) reported the directional movement of cortical pigment granules toward the cleavage furrow in *Arbacia* eggs. Concentration of membrane receptor-bound Con A into the cleavage furrow has been observed with fertilized sea urchin eggs and with cultured macrophages (McCaig and Robinson, 1982; Koppel et al., 1982). Koppel et al. (1982) analyzed the pattern of recovery after photobleaching fluorescent Con A and reached the conclusion that the concentration into the furrow most likely represents directional movement rather than trapping of receptors. As commonly proposed, such directional movement of membrane receptors is probably driven by the movement of underlying cytoskeletal networks (Bourguignon and Bourguignon, 1984).

Our hypothetical model of actin reorganization during cytokinesis is shown in Fig. 6. We propose that there is a continuous recruitment of actin filaments from the cytoplasm into the cortex. The cortical actin filaments then move toward the equatorial plane where they become organized into the contractile ring and may subsequently dissociate from the cortex as suggested by Schroeder (1972). The dissociated filaments may then move away from the equatorial region and reassociate with the cortex, thus completing the cycle. It is noteworthy that a similar process, involving backward cortical flow and cytoskeletal recycling, has long been proposed in polarized interphase cells (discussed in Bray and White, 1988; McKenna et al., 1989). Thus one interesting view is that cytokinesis may represent the establishment of opposite cortical flow in the two daughter cells as they enter interphase.

The source of forces responsible for the cortical flow is unknown. White and Borisy (1983) proposed that the movement may be a result of relaxation at the poles (Schroeder, 1981), which disturbs the balance of forces on a contracting cortex and drives the contractile elements toward the equatorial region. Alternatively, it is conceivable that forces may be generated by interactions between the cortex and the underlying cytoplasm. This interaction may contribute to the process of cytokinesis since counter forces directed toward the poles would be exerted on the cytoplasm, thus driving the cytoplasm in the equatorial region toward the poles and causing the constriction of the cell.

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