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Assembly Of Cytoskeletal Proteins Into Cleavage Furrows of Tissue Culture Cells

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KEY WORDS cleavage furrows; tissue culture cells; actin; myosin; mitotic poles; microtubules

ABSTRACT We review results obtained after fluorescent actin and myosin II probes were microinjected into interphase and prophase PtK2 and LLC-PK tissue culture cells to follow the changing distribution of these cytoskeletal proteins in the live cells during division. The fluorescent probes first begin to assemble into the future furrow region during mid-anaphase before any sign of initial contractions. The total concentrations of F-actin and myosin in the cleavage furrow begin to decrease a few minutes after the onset of furrow contraction. The cell's shape and the position of its mitotic spindle affect the deposition of cytoskeletal proteins in the forming cleavage furrow. In cells with two spindles, contractile proteins were recruited not only to the cortex bordering the former metaphase plates but also to the cortex midway between each pair of adjacent non-daughter poles or centrosomes. The furrowing between adjacent poles seen in these cultured cells are similar to the furrows observed by Rappaport [(1961) J Exp Zool 148:81–89] when echinoderm eggs were manipulated into a torus shape so that the poles of two mitotic spindles were adjacent to one another. These observations on injected tissue culture cells suggest that vertebrate cells share common mechanisms for the establishment of the cleavage furrow with echinoderm cells. *Microsc. Res. Tech. 49:190–201, 2000.*

INTRODUCTION

A number of steps have to be coordinated for the process of cytokinesis to be effective: site determination of the cleavage furrow; recruitment of furrow proteins to the appropriate site; attachment of the actin filaments to the furrow cortex; contraction of the actin-myosin filaments, disassembly of the proteins in the furrow during contraction; midbody formation, sealing, and, finally, severing to produce two independent daughter cells (Dan, 1943; Mabuchi, 1986; Rappaport, 1996; Rappaport 1996; 1997; Sanger, 1975; Sanger et al., 1985; 1989; Schroeder, 1975). Evidence has been obtained by several laboratories that supports the idea that microtubules may influence the flow of surface receptors towards the forming furrow, the recruitment of actin and molecules to the furrow region, and the formation and sealing of the mid-body (Berlin et al., 1978; DeBiasio et al., 1996; Rogalski and Singer, 1985; Waterman-Storer et al., 1993; Wheatley and Wang, 1996; Wheatley et al., 1997; Yonemura et al., 1993; see reviews by Field et al., 1999; Glotzer, 1997; Hales et al., 1999).

Cytokinesis requires the transitory assembly of an F-actin/myosin contractile system, the cleavage furrow, that shares some of the properties of stress fibers in non-muscle cells and of myofibrils in muscle cells (Sanger and Sanger, 1980; Sanger et al., 1994, 1998). Each of the three contractile systems is dynamic and capable of reversible assembly and disassembly in response to a variety of signals (Dabiri et al., 1997, 1999; Kreis et al., 1982; Mittal et al., 1987a). Our work has supported the hypothesis that there is a similar sarcomeric structure between these three contractile systems (Schroeder, 1975; Sanger and Sanger, 1980; Sanger et al., 1994). Two of the molecules involved in attaching the interphase stress fibers to the cell surface (Burridge and Chrzanowska-Wodnicka, 1996; Simon et al., 1991) are also found in cleavage furrows, i.e., alpha-actinin and talin (Dabiri et al., 1999; Sanger et al., 1987, 1994).

One of the goals of current research in the field of cytokinesis is to understand the factors that are responsible for the site-specific assembly of the cleavage furrow. Tissue culture cells, especially epithelial cells, present two great advantages for this type of study. First, in contrast to fibroblasts (and the blastomeres of echinoderms), epithelial cells remain flat during cytokinesis (Sanger et al., 1984) and provide a small optical pathway for the study of fluorescent molecules that can be injected into the cells (Sanger et al., 1980). Second, the epithelial cells often assume a wide variety of shapes. This has permitted us to study the effects of cell shapes and the unusual positions of the mitotic spindles in these cells on the initial deposits of cytoskeletal proteins during cleavage formation (Mittal et al., 1987a; Sanger et al., 1989, 1995, 1998). The site of cleavage furrow assembly is always in the region of the cell cortex that borders the equatorial plane (the former metaphase plate) of the mitotic spindle. It is at mid-anaphase that actin and myosin can first be de-

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tected in this site (Mittal et al., 1987a; Sanger et al., 1989).

In this article we discuss the effect of cell shape, spindle position, and multiple mitotic poles on the assembly of fluorescently labeled actin and myosin probes into the cleavage furrows of tissue culture cell lines. The furrowing results in these vertebrate-derived tissue culture cells are similar to those obtained for the experimentally manipulated echinoderm blastomere cells (Rappaport, 1996). All of these results support the conclusion that there is a common mechanism for the assembly of the furrow in at least echinoderm and vertebrate tissue culture cells where the centrosomes and their linearly connected microtubules may play an important role for the assembly of the cleavage furrow.

MATERIALS AND METHODS

Tissue Culture Cells and Fluorescent Probes

PtK2 and LLC-PK cells, both derived from renal epithelial tissue, remain relatively flat during cell division. They were grown on glass bottom culture dishes and microinjected with fluorescent probes using conditions previously reported (Mittal et al., 1987b; Sanger et al., 1998). Lissamine rhodamine labeled actin was prepared as described (Sanger et al. 1989). This same labeling method was used to tag bovine serum albumin (Sigma, St. Louis, MO). Rhodamine-labeled phalloidin was purchased as a powder from Molecular Probes Inc. (Eugene, OR) and dissolved in methanol at 3.3 μmol. After evaporation of most of the methanol, distilled water was added to yield a final concentration of 5 to 60 μmol (Sanger et al., 1994). Fluorescently labeled myosin light chains were prepared according to the procedures described by Mittal et al. (1987a). The labeled monomer actin, bovine serum albumin, and myosin light chains were microinjected at a needle concentration of 1 mg/ml during either interphase (monomer actin, bovine serum albumin, myosin light chains, or phalloidin) or in prophase (fluorescently labeled phalloidin) using a specially designed pressure injection device coupled to a Leitz micromanipulator (Pochapin et al. 1983; Sanger et al., 1985).

Imaging of the Dividing Cells

The microscope stage was kept at a temperature of 37°C. and 5% CO_2 was perfused over the culture dish during observations and image recording. A Dage-MTI-SIT camera (Dage-MTI Inc., Michigan City, IN) with gain and black level set to manual and a 63× Zeiss phase-contrast plan apochromat objective (N. A. 1.3) were used to record the low light images. The images were processed with an Image-I processing system (Universal Imaging Inc., West Chester, PA). Fifteen to thirty frames were summed to obtain a recorded image for a single time point. A background image was subtracted before intensity measurements were made. Some of the images were filtered to enhance the fibers in the cells. To obtain relative measurements of the amount of F-actin and myosin II at different timepoints during cell division we used the following procedures. The images were recorded at the same position and focus throughout the experiments and a background image of an adjacent field lacking fluorescence was subtracted from each timepoint. The Area Measurement function was used to record the fluorescence intensity within boxes of equal size placed over regions of the injected cells (cleavage furrows, mitotic poles, interphase cortices) and over adjacent uninjected cells. Intensity was plotted against time during cell division to determine the changing amounts of F-actin or myosin II in a particular region of the cell. Images were recorded from the monitor by photographing the screen of the monitor (Waterman-Storer et al. 1993) or by assembling digitized images using Photoshop (Adobe Systems, Mountain View, CA; Turnacioglu et al., 1998).

RESULTS and DISCUSSION

Recruitment of Actin and Myosin II Into the Cleavage Furrow in Cultured Cells

Cultured epithelial cells like PtK2 and LLC-PK, which remain relatively flat during mitosis, can provide a window for monitoring the dynamic distribution of some of the proteins involved in cell division. Trace amounts of fluorescently labeled proteins and probes, introduced into the cells via microinjection, localize with the endogenous proteins and mark the sites of concentration in the live cell (Taylor and Wang, 1980). Variation in the relative fluorescence intensity of the protein as the cell divides, provides a picture of the protein's changing distribution in the dividing cell. Rhodamine phalloidin, for example, binds only the polymerized form of actin (F-actin) and its injection into cells (Wehland and Weber, 1981) allows the recruitment of F-actin to be followed, beginning in prophase, to the future furrow region (Fig. 1). It is absent from the spindle and central part of the cell at metaphase, then beginning at mid-anaphase, a low level of F-actin can be detected in the cortex over the midzone of the spindle. In images of timepoints taken of the middle of an injected, symmetrically dividing cell in Figure 1, F-actin was recorded as it assembled in small, fine fibers in the furrow region (timepoints nos. 2 and 3) growing to a band that maintained a width of about 10 μm as contraction proceeded and the midzone decreased in diameter (timepoints nos. 4–16). We have found that fluorescent probes for both actin and myosin assemble in a band in the furrow that remains at a constant width of about 10 μm during contraction in large cultured cells (Mittal et al., 1987a; Sanger et al., 1989, 1994, 1998).

An examination of the fluorescence intensity profile of phalloidin in the furrow region of an injected LLC-PK cell (Fig. 2) can reveal the time course of F-actin assembly with respect to mitotic stage (Fig. 2). As the injected LLC-PK cell progressed from metaphase to telophase (Fig. 2), the fluorescence intensity was recorded from uniformly sized regions superimposed over the mitotic poles, furrow zone, and neighboring injected and uninjected interphase cells. Setting metaphase at time 0, the measurements showed a steady accumulation of F-actin fluorescence in the forming furrow region during anaphase and telophase with the level of actin fluorescence continuing to increase after the onset of furrowing. The actin concentrations near the two mitotic poles were unchanged near one pole and decreased steadily near the opposite pole, indicating that the actin recruitment to the furrow does not always proceed symmetrically. In other

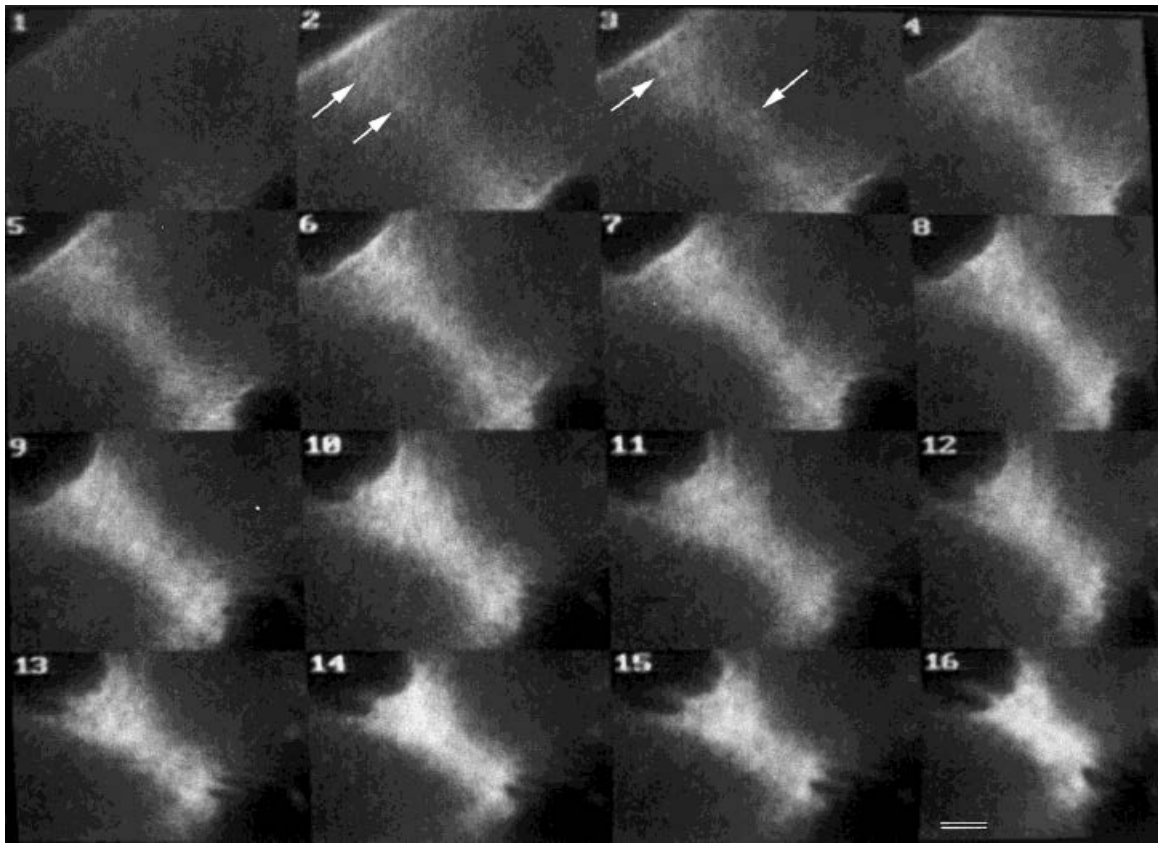
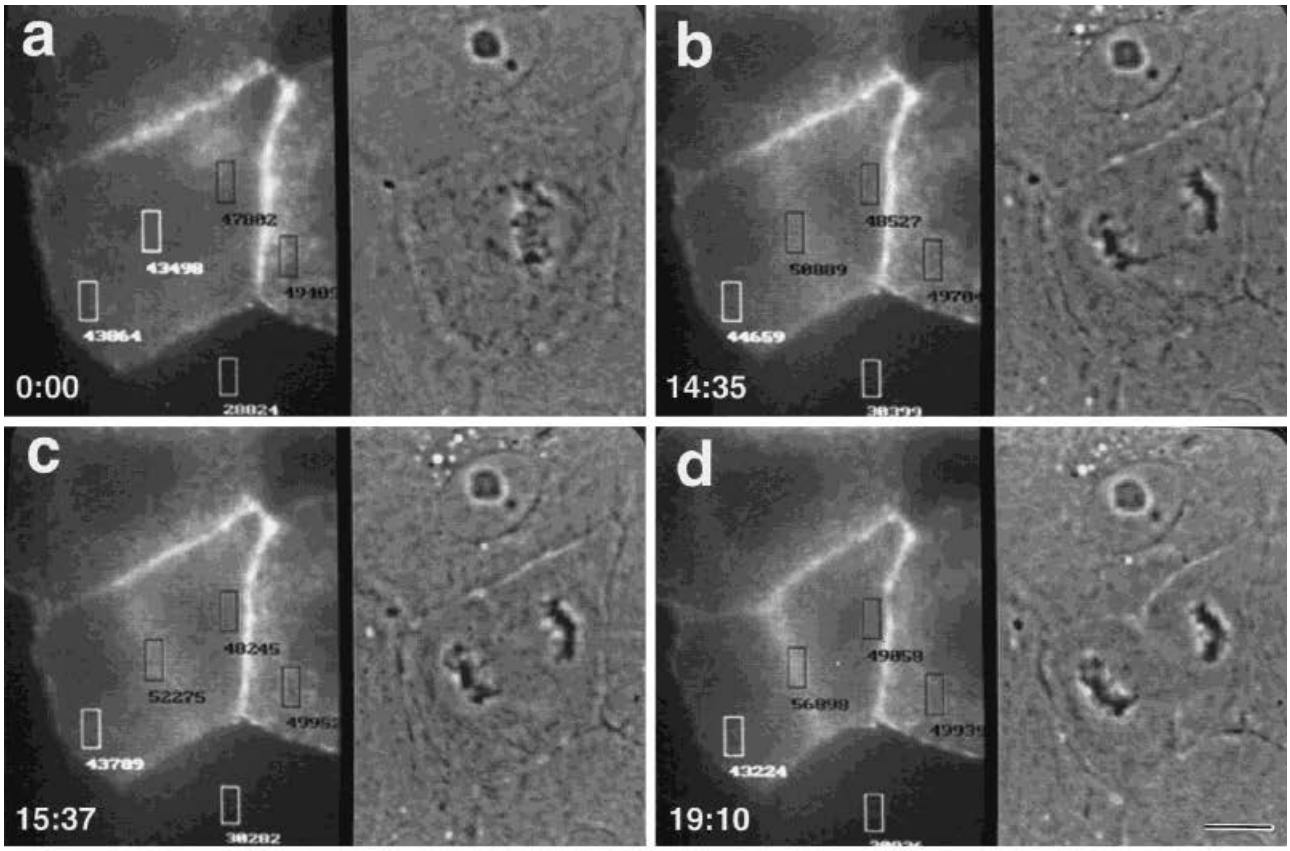


Fig. 1. Time points of the middle of a PtK2 cell undergoing cytokinesis (image numbers 1 to 16) following microinjection with rhodamine labeled phalloidin, a compound that binds to F-actin. The focus is on the ventral surface of the dividing cell. The F-actin can be detected first in the future furrow region is in small patches (images 2,3). Additional F-actin is recruited to the furrow region that obscures the original patches (image numbers 4–7). The F-actin filaments move closer together during contraction of the furrow region (images 4–16). These sixteen images were recorded 1 minute apart starting at mid-anaphase (image number 1). Scale = 5 μm.

cases of injected LLC-PK and PtK2 cells (not shown), while the level of F-actin fluorescence in the furrow region always increased steadily as the furrow assembled and contracted, the actin concentrations near the poles would either decrease or remain unchanged. In cases where there was a decrease of F-actin concentration near the poles, a plateau was reached during the contraction of the furrows. The continual increase in actin fluorescence as the furrow contracts corresponds to measurements made in a small subregion of the furrow, and may reflect increased interdigitation of actin filaments that are arranged in sarcomere-like arrays in the cleavage furrow (Sanger and Sanger, 1980; Sanger et al., 1994; Schroeder, 1975).

To measure the level of F-actin recruited to the entire furrow zone, an area of measurement encompassing the furrow region was used to record fluorescence in the furrow region from mid-anaphase to mid-telophase (Fig. 3a–d). F-actin accumulated in the furrow as it formed and continued to increase for about 2 minutes after contraction of the furrow was visible with phase-contrast optics (Fig. 3, graph). The time course of loss of F-actin in the furrow region coincided, in this particular cell, with the level of actin near the poles returning to a constant value after previously decreasing during furrow formation. Although the fluorescence in a small subregion of the furrow continued to increase in intensity through cytokinesis (Fig. 2), measurement of the entire furrow region indicates that F-actin begins to leave the furrow within a few minutes after the initiation of furrowing.

Similar measurements of myosinII in the full furrow region reveal a profile mimicking that of actin (Fig. 4). Interphase cells cells injected with fluorescent myosin light chains incorporate this probe into the myosin filaments of both muscle and non-muscle cells (DeBiasio et al., 1996; Mittal et al., 1987a; Sanger et al., 1989). When PtK2 cells, injected with fluorescent myosin light chains, entered cell division the fluorescence was measured across the mid-zone of the cell as division progressed from metaphase to the end of cytokinesis. Figure 4 shows the changing amount of myosin from metaphase to the end of cytokinesis in such an injected cell. During anaphase before signs of furrow contractions, there was an accumulation of myosin in the future furrow region that continued for two and a half minutes after the initiation of furrow contraction. DeBiasio et al. (1996) have reported a similar accumulation of myosin II at anaphase. While furrowing proceeded (Fig. 4), there was a sharp drop in the amount of myosin in the contracting furrow region. These results indicate that both myosin and F-actin filaments assem-



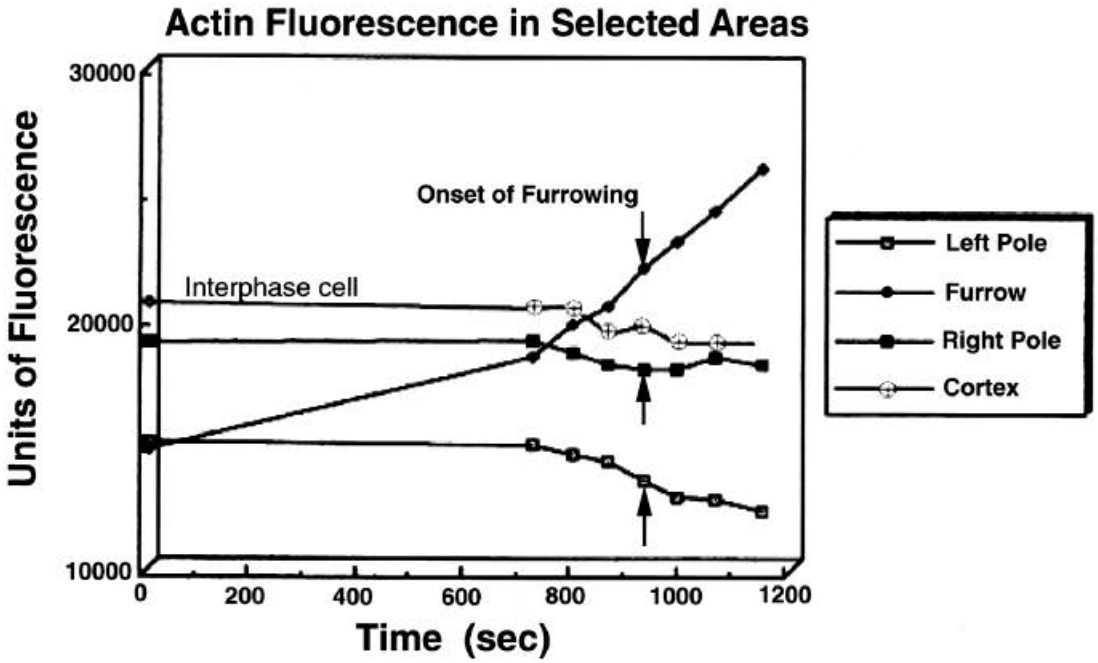


Fig. 2. An LLC-PK cell injected with rhodamine phalloidin. **a–d:** Four time points of paired fluorescent and phase-contrast images collected from metaphase through mid-cytokinesis are shown here. Actin fluorescence was measured in small uniformly sized areas at each pole, within the future cleavage plane and in a phalloidin-injected interphase cell. Note the lack of concentration of F-actin in the metaphase mitotic spindle (a). The times listed are from the initial metaphase recorded image in a. Scale = 10 μm. Graph of the measurements of fluorescence of rhodamine phalloidin in different areas of the mitotic and interphase LLC-PK cells illustrated above. The fluorescence of the F-actin probe increased within the forming furrow before the onset of cytokinesis and continued rising during cytokinesis. During this time there was a small decline in the level of fluorescence near one pole and a nearly constant level near the other pole. As a control, the fluorescence in a neighboring interphase cell was measured and found to be relatively constant as well.

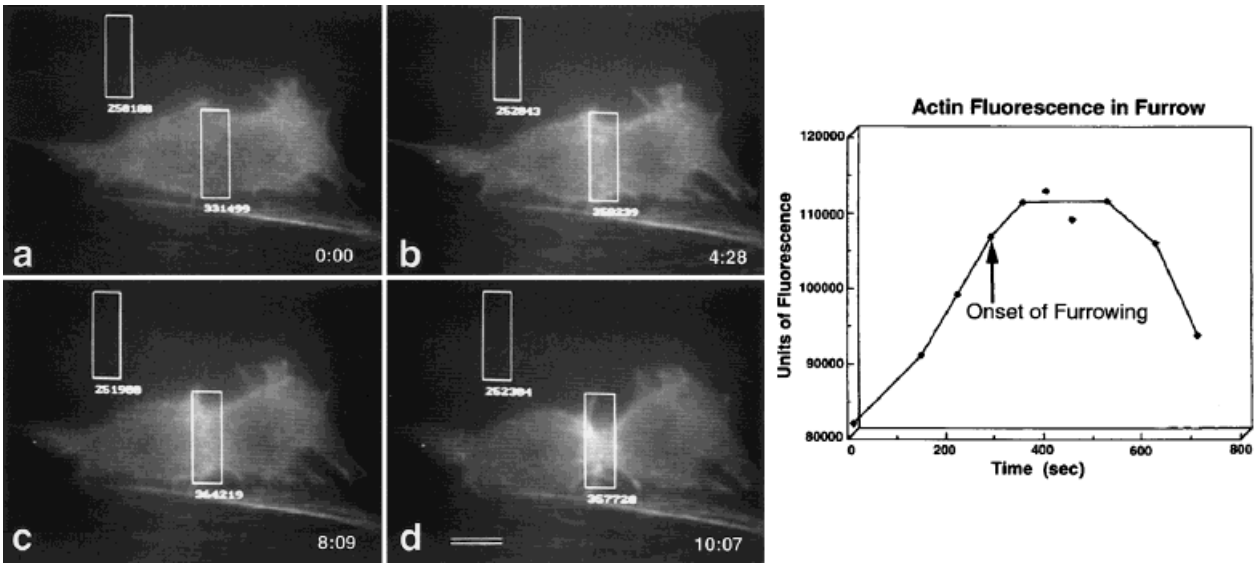


Fig. 3. Images of a dividing PtK2 cell microinjected with rhodamine phalloidin. The level of actin recruited to the entire furrow zone was analyzed by recording fluorescence in the furrow region. The total furrow fluorescence of the F-actin probe was measured from mid-anaphase to mid-telophase using an area of measurement that encompassed the entire furrow region. The same sized area was measured over an uninjected cell to detect any changes in the background. The time points listed on the images (**b–d**) are the minutes and seconds from the first image in **a**. Scale = 10 μm. Graph of the measurements of fluorescence of rhodamine phalloidin in the furrow region of the PtK2 cell illustrated above. There was a gradual increase in the amount of F-actin probe fluorescence before there was any sign of furrow contraction. F-actin continued to accumulate in the furrow for 2 minutes after the initial furrow contraction and decreased as furrowing continued to completion.

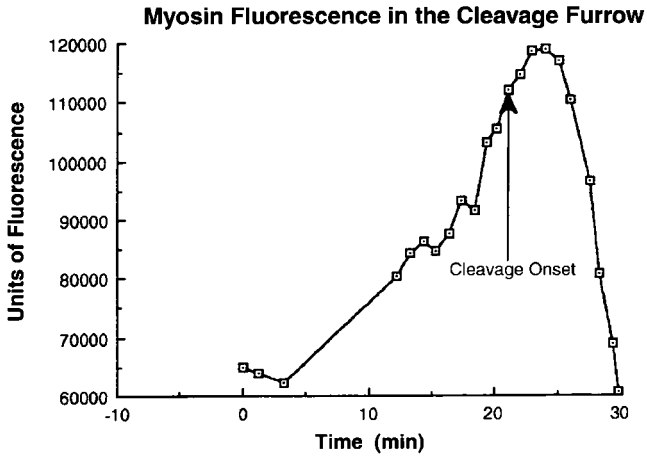


Fig. 4. Measurements of fluorescence of rhodamine labeled myosin light chains in a dividing PtK2 cell. Fluorescence was measured over an area that covered the entire furrow region. During metaphase (time 0) there was no change in fluorescence in the future furrow zone. During anaphase, myosin fluorescence began to increase in that area before any indication of surface contractions. Myosin fluorescence continued to accumulate in the furrow region for 2.5 minutes after the initial contractions were visible, and then decreased in the contracting furrow as cytokinesis progressed to completion.

ble in the future furrow region during early anaphase before any visible signs of contraction, and begin to disassemble within a few minutes after the initial furrow contractions. We never observed an equatorial accumulation of F-actin or myosin fluorescence in metaphase cells, even in cases where a cell remained in metaphase for an hour before anaphase began. The signal for this surface instruction for furrow formation must be transmitted soon after the end of metaphase since the actin and myosin molecules arrive at the future furrow region at mid-anaphase, a time period of 2 to 4 minutes after the metaphase/anaphase transition.

F-actin and myosin normally begin to leave the furrow region within minutes after the initiation of contraction (Figs. 3 and 4). In cells with furrows that do not completely encircle the cell, however, the proteins remain for up to 1 hour before disassembling from the multinucleate cell. In some injected cells delayed in cytokinesis, we found that fibers remained in position after the furrow relaxed and stress fibers formed in the cytoplasm of the binucleate cell (Sanger et al., 1998).

Figure 5 illustrates an irregularly shaped PtK2 cell with an incomplete furrow that was previously injected with fluorescently labeled monomeric actin during interphase. It is connected to nearby uninjected cells. The microinjection of rhodamine labeled monomeric (G) actin, in contrast to rhodamine phalloidin, allows both polymerized and unpolymerized actin molecules to be followed in dividing cells (Sanger et al., 1989). To the left of the spindle, the cytoplasm extended 30 μm along the middle of the cell. Fluorescent actin began to accumulate in the future furrow region during mid-anaphase; however, in contrast to the circular cleavage furrow illustrated in Figure 1, the furrow in this cell did not extend all the way across the diameter of the cell. The incomplete ring contracted, but did not constrict the cell into two daughter cells. Stress fibers began to reassemble in the presence of the furrow and

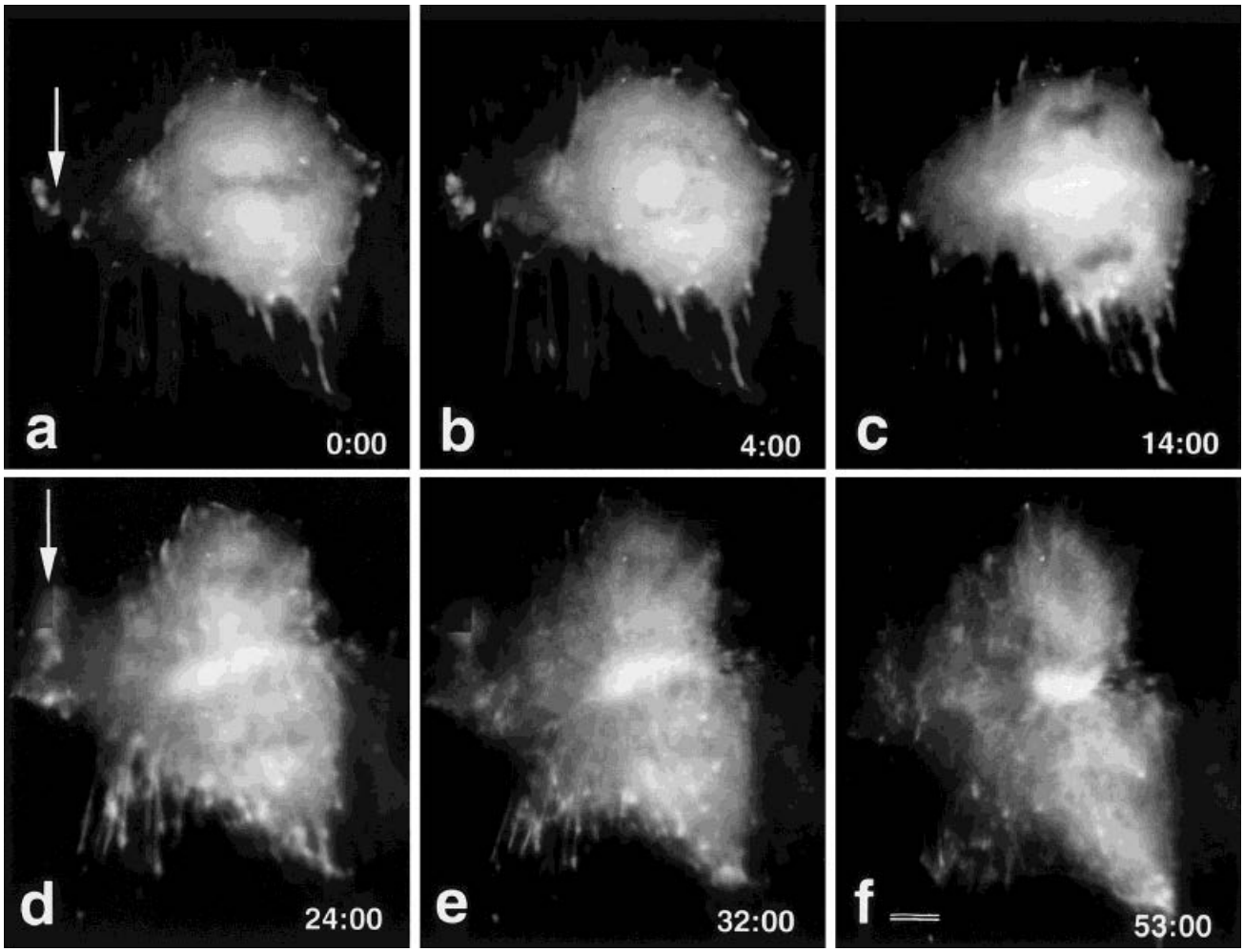


Fig. 5. Actin recruitment to the cleavage furrow in a PtK2 cell with an unusual cell shape. Monomer actin labeled with lissamine rhodamine was injected into the cell during interphase. When the spindle formed, it was positioned adjacent to a broad area of cytoplasm (arrows in a and d) that extended 30 μm from the left side of the metaphase mitotic spindle. The times listed are from the initial metaphase recorded image in a. **a:** Image taken 2 minutes before the initiation of anaphase. The focus was on the bottom side of the cell. The chromosomes on the metaphase plate were visible as a dark image. Fluorescent actin was concentrated in the mitotic spindle. **b:** Four minutes later the two sets of chromosomes were visible in mid-anaphase position. **c:** Ten minutes later a band of actin stretched from one edge of the cell but did not reach the edge of the extended flap of cytoplasm. d: Ten minutes later active furrowing had occurred in the furrow where the actin band was localized, but furrowing did not extend to the left side of the cell. Furrowing continued on one side of the cell for an additional (**e**) 8 and (**f**) 21 minutes after the time point in d. The delayed time of this cytokinesis led to the formation of stress fibers in both parts of the cell while furrowing was still active. The actin in the furrow region eventually disassembled and a binucleated cell formed. Scale = 10 μm.

the constriction eventually relaxed. As a rule when the band of contractile protein in injected cells reaches only part way across dividing cells, cytokinesis fails to go to completion and form two dauighter cells. Injected cells like this demonstrate that a circular ring of filaments (Schroeder, 1975) is not required to produce contractions in the furrow region, but it is required for cytokinesis to proceed to completion and form two daughter cells in these epithelial cell cultures.

Results similar to those described above with actin probes are also obtained when myosin probes are injected into PtK2 cells that undergo cell division. In cells where the spindle is in the middle of the cell, a circular ring of myosin is formed (Mittal et al., 1987a; Sanger et al., 1998). However, in oddly shaped cells or those with asymmetrically positioned mitotic spindles, atypical cleavage furrows form. In the example in Figure 6, the spindle was displaced to one side of the injected cell. As in actin probe-injected cells, myosin began to accumulate into the future cleavage furrow during mid-anaphase. Initially a punctate fluorescent pattern of myosin could be seen in the forming furrow, but as more myosin accumulated this substructure was not visible. In this cell, as is true for all injected cells with asymmetrically positioned spindles, the proteins first assembled along the cell surface that was closest to the mitotic spindle. This area was also the first area of the cleavage furrow to contract, and the amount of myosin was greater on this side. Since the myosin band extended completely across the midsection of the cell, the

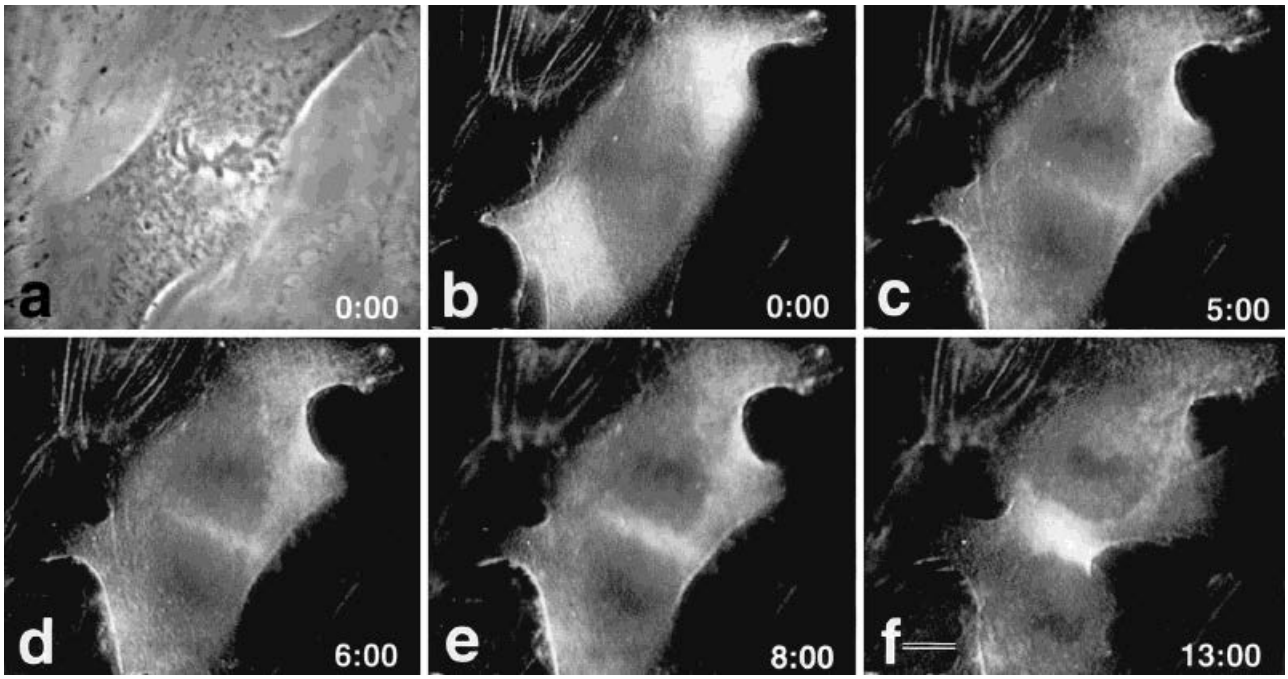


Fig. 6. Time points of a PtK2 cell undergoing cell division after microinjection during interphase with fluorescently labeled myosin light chains. **a,b:** Phase and fluorescent images of the metaphase cell. Note the asymmetrical position of the mitotic spindle and the absence of a concentration of fluorescent myosin in the mitotic spindle. The times listed are from the initial metaphase recorded image in a and b. **c:** Five minutes after the initiation of anaphase, myosin was detected between the separating chromosomes near the side of the cell that was closest to the edge of the mitotic spindle. **d,e:** One and two minutes later (6 and 7 minutes post-anaphase) the band of myosin extended toward the opposite side of the cell. The concentration of myosin remained higher on the side where it first assembled, and contraction began on that side (e). **f:** Five minutes later (12 minutes post-anaphase), the band of myosin was thicker and contraction progressed from both sides of the cell. Scale = 10 μm.

cleavage ring contracted down to a midbody to form two daughter cells.

When cytokinesis goes to completion in injected cells, two compact rings of actin and myosin can often be seen at each end of the midbody that connects the two daughter cells (Sanger et al., 1989). The microtubules in the midbody are distinguished by a concentration of gamma-tubulin (Julian et al., 1993; Shu et al., 1995), but its role in midbody function is not known. It is not clear how the single cleavage band of actin and myosin is realigned into these two smaller bands. It is also puzzling that the two stable rings form in a site where active disassembly of actin and myosin filaments has just finished (Sanger et al., 1989, 1994, 1998). These bands of contractile protein may play an important role in separating the two daughter cells by maintaining the cell surface membranes in close proximity in the midbody until they seal (Pochapin et al., 1983; Sanger et al., 1985). Interference with actin polymerization often leads to the relaxation of the furrow at the midbody stage (Sanger and Holtzer, 1972; Sanger et al., 1995).

In previous experiments we tried to block cytokinesis by the microinjection of myosin II antibodies (Zurek et al., 1990) or thymosin beta4, a monomer actin binding protein, (Sanger et al., 1995) into tissue culture cells. In both cases, cytokinesis was greatly delayed and failed to reach completion, but contraction nevertheless occurred in the furrow for up to an hour. This is in marked contrast to the myosin antibody injection experiments of embryonic blastoderm cells in sea urchins (Mabuchi and Ohno, 1977) where cytokinesis contraction was blocked and a binucleate cell was formed within the normal time of the mitotic cycle of the embryonic cells. In contrast to the 24-hour mitotic cycle of the PtK2 and LLC-PK tissue culture cells, echinoderm blastoderm cells divide every 30 minutes, and the signal to disassemble contractile proteins in the furrow region may be more tightly controlled by time in the embryonic cells. It is as if the signal to disassemble the cleavage furrow in tissue culture cells can be delayed by some factor present (or absent) if contraction is greatly extended. Since microtubules may target contractile proteins to the furrow and are required for furrow completion (Sanger et al., 1998; Wheatley and Wang, 1996), it will be of interest to determine if microtubules are associated with these delayed cleavage furrows.

Recently, Matsumura et al. (1998) have reported immunofluorescence data that show that phosphorylated myosin light chains are present in the forming furrow region. Other proteins that are known to regulate the phosphorylation of these light chains have also been detected in the forming furrow regions as well: Rho (Takaishi et al., 1995), Rho-kinase (Kosako et al., 1999), and the myosin binding subunit (MBS) of myosin phosphatase (Kawano et al., 1999). It is known, in fact, that reagents that interfere with the normal func-

tions of the Rho/Rho-kinase pathway inhibit cytokinesis (Kishi et al., 1993; Mabuchi et al., 1993; Yasui et al., 1998). What remains to be determined is how the timing of the regulation of myosin filament assembly and disassembly is controlled during cytokinesis (Fig. 4). In the myosin injection experiments of Zurek et al. (1990), the cells were able to prolong the time it took to complete cytokinesis. In the next section on unusually shaped cells and its effect on cytokinesis, we present data that indicate some feedback system exists in these cleaving cells to regulate how long myosin can function in the furrow region.

Position of the Cleavage Furrow in Cultured Cells

It is clear that in animal cells the site of cleavage furrow assembly is linked to the position of the mitotic spindle (reviewed in Rappaport, 1996). In dividing, cultured cells, the metaphase spindle can slowly reorient by as much as 90° before anaphase is initiated, and as expected, the final position of the mitotic spindle at the initiation of anaphase determines where the contractile proteins will be deposited to assemble the cleavage furrow. The signal for site selection occurs within the first few minutes of anaphase, judging from the time labeled F-actin or myosin is first detected at the site where the furrow will form (Mittal et al. 1987a; Sanger et al., 1989, 1994, 1998).

In examples where more than one spindle is present in the injected cells, furrows can also form in the regions between adjacent mitotic poles, provided they are not too far apart. Rappaport first reported these extra furrows forming between adjacent mitotic poles in echinoderm blastomeres (Rappaport, 1961). He predicted that the centrosomes exerted some influence on the cell surfaces between these poles. Over the next 35 years he has presented many different types of ingenious experiments to confirm and extend these results; this work is reviewed in his recently published book (1996).

In an elegant set of experiments combining biochemical and morphological manipulation of echinoderm blastomeres, Shuster and Burgess (1999) arrested mitosis by the injection of a nondegradable cyclin B. The mitotic spindle formed and anaphase began, but the mitotic poles moved apart only slightly and furrowing did not occur. When two glass needles were used to push the two asters of the arrested spindle close to the cell surface a furrow formed 5 minutes later. Shuster and Burgess (1999) suggest that a cleavage stimulus is delivered to the cell cortex via astral microtubules. In cases where the asters in the injected cells were too far apart, i.e., greater than 45 μm, no furrow was formed in the manipulated cells agreeing with previous measurements by Rappaport (1996) in manipulated echinoderm blastomeres.

Large cultured cells like PtK2 are often multinucleated and can make ideal subjects for examining the relationship between mitotic poles and cleavage furrow assembly to determine if it mirrors the relationship found in echinoderm blastoderms. Following live cells microinjected with fluorescent actin or myosin probes allows the distribution of cytoskeletal proteins in the furrow to be correlated with the position of multiple mitotic poles. Figure 7 shows two mitotic spindles that were present in the same large cell that measured 100 by 50 μm, and had been microinjected with rhodamine phalloidin during prophase. This cell also had the added unusual feature that the two mitotic spindles assembled closer to one side (left) of the large cell. F-actin accumulated midway between the two sets of separating chromosomes and also between the two sets of mitotic poles of adjacent spindles. The two fluorescent bands extending across the two former metaphase plates were connected to one another by a less concentrated band of actin filaments with the band closest to cell margin contracting that side of the cell inward while the second band extended only part way across the cell to the right (Fig. 7). The two F-actin bands running vertically between the two sets of adjacent mitotic poles merged with the mid-spindle bands, and in one case, between the lower two poles, reached the cell edge, which was pulled inward. The actin band that formed between the upper set of adjacent mitotic poles did not extend to the upper edge of the cell. Additional timepoints showed that contraction occurred in the bands of F-actin fibers in these four regions but the cell did not form four daughter cells.

In other binucleate cells that entered mitosis with two mitotic spindles, we found that injected actin or myosin probes also assembled between adjacent poles of neighboring mitotic spindles as well as between the separated chromosomes. If the two adjacent poles were too close or if they were far apart in the cytoplasm, the fluorescent probe did not localize between the adjacent poles. This is similar to the relationships uncovered by Rappaport (1961) through experimental manipulation of echinoderm blastomeres.

The idea that the interaction of astral spindle fibers with the cell surface was required for furrow formation was suggested by experiments of Dan (1943) who created perforations in different locations adjacent to the mitotic spindle of echinoderm cells. When holes were placed between one side of the spindle and the cell surface furrowing was blocked between the cell surface and the perforation, contraction occurred on the side of the perforation close to the mitotic spindle. Cao and Wang (1996) were able to produce similar perturbations between the cell surface and the central part of the spindle in cultured cells and in addition, they localized the microtubules in the manipulated cells. Blocks introduced near the spindle midzone also inhibited normal cytokinesis, leading to the proposal that a signal from the microtubules in the spindle midzone was required for furrowing, as opposed to a requirement for astral or polar microtubules.

Recent experiments based on the clever idea of fusing cultured PtK1 cells to create multi-spindle cell divisions (Rieder et al., 1997; Savoian et al., 1999), also showed that ectopic furrows formed between two adjacent mitotic spindles. In furrows that completed contraction, the midbody contained bundles of microtubules, the chromosomal protein INCENP, and CHO1, a member of the kinesin superfamily. In furrows that regressed, microtubules were loosely organized and INCENP and CHO1 were absent. The interpretation of Savoian et al. (1999) that bundles of microtubules, INCENP, and CHO1 are necessary for the formation of the midbody is supported by experiments in *Caenorhabditis elegans.* Mutants of *C. elegans* lacking ZEN-4, a homologue of CHO1, can initiate furrowing

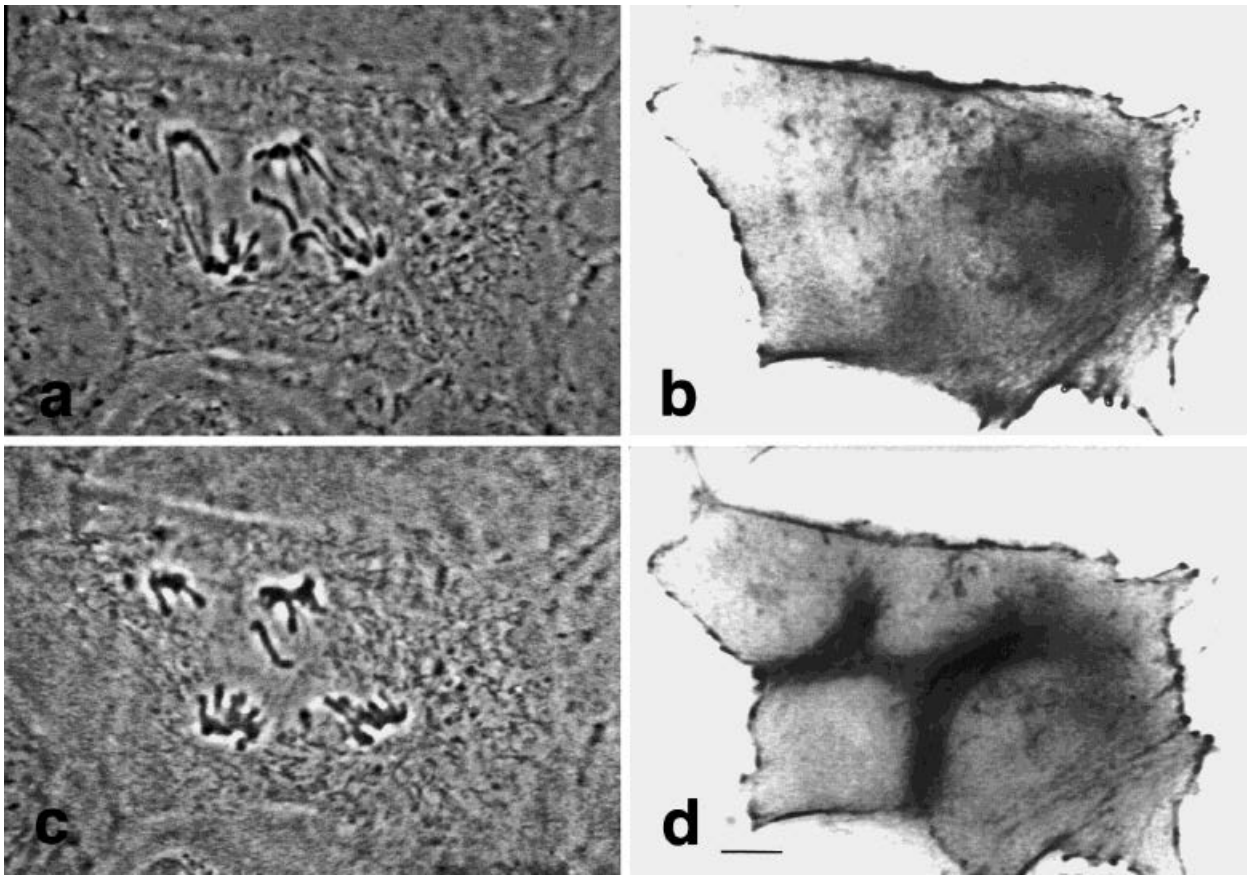


Fig. 7. Two time points of a PtK2 cell with two mitotic spindles, previously injected with rhodamine-labeled phalloidin. The positions of the chromosomes can be seen in the phase-contrast images (**a,c**) and the position of phalloidin-labeled F-actin in the reverse contrast images (**b,d**). The center of the rightmost spindle was 40 μm from the right side of the cell while the center of the left spindle was 15.5 μm from the left edge of the cell. The actin was recruited to the positions of the two former metaphase plates and in the areas between the two pairs of adjacent poles (d). Because the two spindles were positioned asymmetrically in the cell, the cleavage furrow that extended across the spindle equators did not reach the most distant side of the cell. The images in a and b are separated from c and d by 7 minutes and 25 seconds. Scale = 10 μm.

but are unable to complete cytokinesis and form midbodies (Raich et al., 1998). These experiments indicate that the bundles of microtubules and microtubule associated molecules (INCENP, CHO 1, ZEN-4), although required for the formation of the midbody, are not involved in the initiation of the future furrow.

The role of microtubules in initiating site-specific assembly of the cleavage furrow could be due to transport of signal molecules along microtubules to the cell cortex or the microtubules themselves might interact with molecules of the actin cytoskeleton in the cortex. Kaverina et al. (1998) have demonstrated recently that microtubules can target to early focal adhesions. These focal adhesions in spreading interphase cells capture and stabilize microtubules. Perhaps there is a complementary relationship between the stable interpolar spindle microtubules and proteins that attach the barbed ends of the furrow actin filaments to the cell surface. Figure 8 offers a model of such a scenario. The concentration of interdigitating microtubules from the two centrosomal regions could interact with the peripheral cell surface and nucleate an assembly of proteins that link membrane, actin cytoskeleton, and microtubules (Sanger et al., 1998).

A circumferential array of the ends of interdigitating microtubules from opposite poles in the former metaphase plate region would provide a circular template for islands of actin filaments to assemble in membrane-associated units comparable to the Z-bands of myofibrils or the dense bodies of stress fibers (Dabiri et al., 1997; Mittal et al., 1989; Rhee et al., 1994). Interaction of myosin filaments with the actin filaments would produce linked contractile units (Fig. 8a, insert 1 and 2). The initial deposits of myosin (Sanger et al., 1994) and actin filaments (Fig. 1) sometime display a punctate pattern, and in dividing cardiomyocytes, there is a distinct beaded pattern of alpha-actinin in the cleavage furrows (Dabiri et al., 1999). Sanger et al. (1995) demonstrated that the injection of thymosin beta4, a monomeric actin binding protein, into early prophase to early anaphase cells resulted in prolonged times for cytokinesis. The rate of furrowing was at a slower rate and there was a greatly reduced amount of fibrous actin in the furrow region. A banded pattern of actin was visible during the early assembly of the furrow region in these injected cells (Sanger et al., 1995). These punctate and banded patterns of F-actin, myosin, and alpha-actinin are consistent with the sarco-

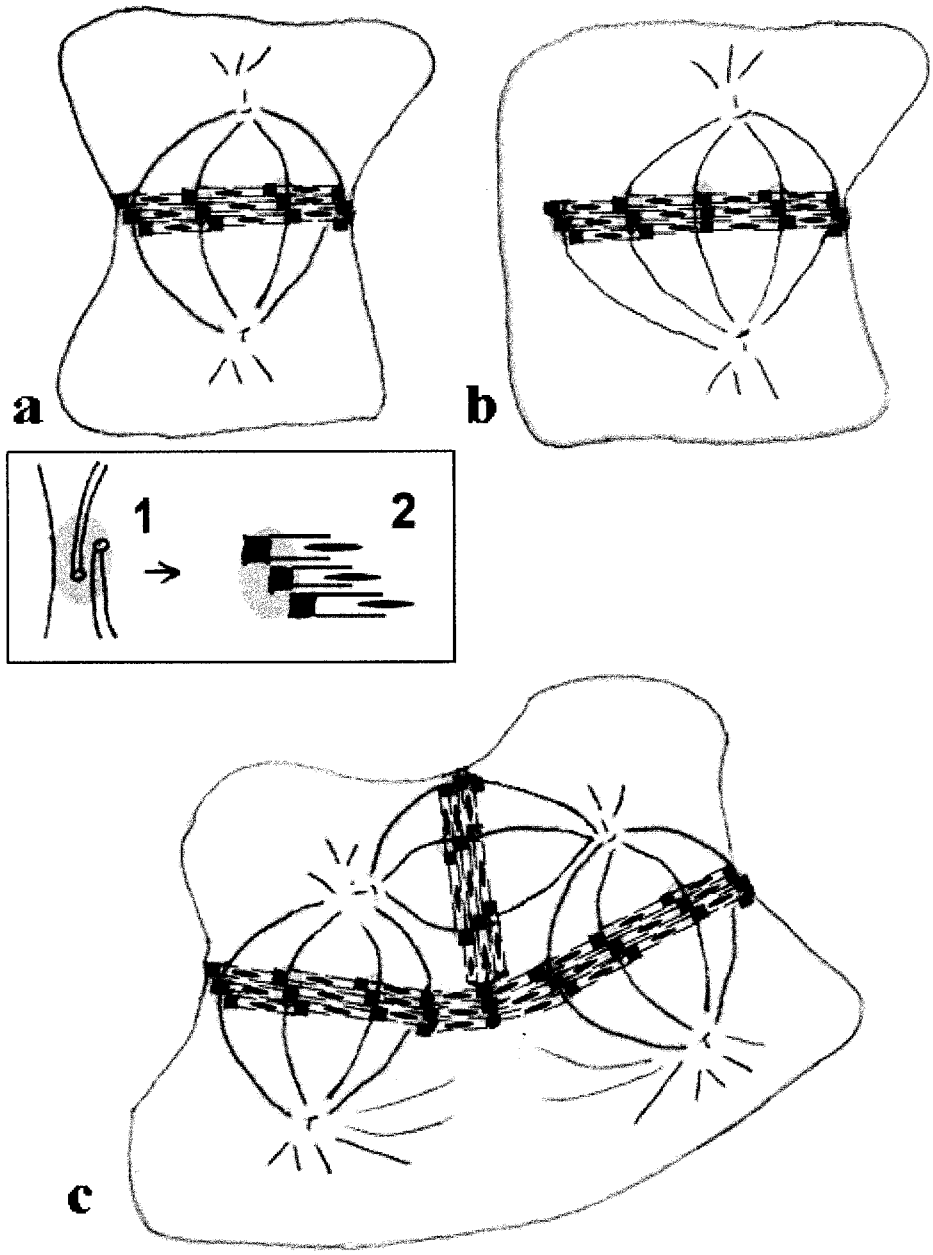


Fig. 8. A model based on the hypothesis that concentrations of centrosomal microtubules interact with the cell surface to stabilize clusters of transmembrane and membrane-associated proteins that bind actin filaments. In turn, the actin filaments interact with myosin filaments to form contractile units. Diagrams of cells show the postulated relationship between microtubules, represented by thin lines, and contractile proteins shown as interconnected banded units. **a:** In cells where the mitotic spindle is symmetrically positioned, the microtubule ends are predicted to target a circular array of contractile protein, i.e., a purse string. The area where the microtubules meet the cell surface is enlarged in the **inset** below a to show a concentrated focus of microtubules (diagrammed as two overlapping ends, **inset 1**) stabilizing a cluster of membrane-associated proteins (gray oval) that interact with a unit of contractile protein (**inset 2**.). The square black boxes represent the proteins that bind the barbed ends of the actin filaments shown in a half sarcomere interdigitating with myosin filaments. **b:** In cells where the spindle is asymmetrically positioned, the microtubules would not reach the distal cell cortex, resulting in the assembly of an incomplete cleavage furrow. **c:** In cells containing two spindles, contractile protein would assemble in the cortex above and below the spindle midzones and between adjacent centrosomes where the required concentration of microtubular ends contacted the cortex. This requisite concentration would not be found between centrosomes that were too far apart or too close together.

meric models for the structure of the furrow region diagramed in Figure 8 (a–c).

Where mitotic spindles were asymmetrically positioned in the cell and the microtubules unable to contact the cell perimeter, an incomplete cleavage furrow would assemble (Fig. 8b,c). The absence of concentrations of microtubules at the edges of these cells would result in a lack of actin filament islands at the cell periphery and the formation of an incomplete furrow. Disassembly of microtubules could lead to the loss of previously formed contractile units, and may explain why concentrations of actin and myosin become diminished in some multipolar cells (Sanger et al., 1998). Wheatley and Wang (1996) have shown that in cells where cleavage furrows relax, bundles of spindle microtubules are absent.

Potential candidates for molecules that could bind the ends of microtubules and interact with actin or actin-binding proteins in the forming furrow region include septins (Glotzer, 1997; Kinoshita et al., 1997), MAPS (microtubule-associated proteins; Pedrotti et al., 1994), and/or motors that move along microtubules (Williams et al., 1995). There may be additional molecules of this category that are not involved in the specification of the furrow site but are necessary for the formation of the midbody to form two separate daughter cells. One such example appears to be the CYK-1 protein of *C. elegans* (Swan et al., 1998). This protein, a member of the formin family, associates with the furrow near the end of telophase. Mutant cells lacking this gene product are able to assemble actin and myosin molecules to form a contractile furrow that does not

contract completely to form a midbody, but relaxes, and a binucleate cell results.

PERSPECTIVE

Cultured epithelial cells, especially PtK2 cells, provide an excellent system for microscopical studies of cytokinesis in live cells We have been able to determine that F-actin and myosin accumulate in the future furrows shortly after the initiation of anaphase and before any sign of surface furrowing. Our localization data, derived from immunofluorescence, microinjection of fluorescently labeled proteins, and transfections of cells with pGFP-alpha-actinin, are consistent with a sarcomeric arrangement of the contractile proteins in the furrow region (Schroeder, 1975; Sanger and Sanger, 1980). Our article supports the views of Rappaport (1996) on the importance of the asters and microtubules in determining the assembly of a contractile ring in mitotic cells.

Advances in camera technology, image processing, and new fluorescent probes should provide additional approaches for the study of cytokinesis in tissue culture cells. A number of proteins involved with different aspects of cytokinesis are being identified in genetic analyses of yeasts and *C. elegans* (Field et al., 1999). It will be exciting to investigate the homologues and roles of these proteins in tissue culture cells. Almost all studies on the role of myosin in cytokinesis have concerned myosin II. There is a growing number of unconventional myosins (Mermall et al., 1998). Myosin I has been localized to midbody region (Breckler and Burnside, 1994). Future work will determine if these other types of myosins are present in the forming furrow region and what roles they have.

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REFERENCES

Berlin RD, Oliver JM, Walter RJ. 1978. Surface functions during mitosis I: phagocytosis, pinocytosis, and mobility of surface-bound con-A. Cell 15:327–341.

Breckler J, Burnside B. 1994. Myosin 1 localizes to the midbody region during mammalian cytokinesis. Cell Motil Cytoskeleton 29: 312–320.

Burridge K, Chrzanowska-Wodnicka M. 1996. Focal adhesions, contractility and signaling. Ann Rev Cell Dev Biol 12:463–519.

Cao LG, Wang Y-L. 1996. Signals form the spindle midzone are required for the stimulation of cytokinesis in cultured epithelial cells. Mol Biol Cell 7:225–232.

Dabiri G A, Turnacioglu KK, Sanger JM, Sanger JW. 1997. Myofibrillogenesis in living embryonic cardiomyocytes. Proc Natl Acad Sci USA 94:9493–9498.

Dabiri G, Turnacioglu K, Ayoob J, Sanger JM, Sanger JW. 1999. Transfections of primary muscle cell cultures with plasmids coding for GFP/BFP linked to full length and truncated muscle proteins. In: Sullivan KF, Kay SA, editors. Green fluorescent proteins methods in cell biology, vol. 58. New York: Academic Press, p 239–260.

Dan K. 1943. Behavior of the cell surface during cleavage V. Perforation experiment. J Faculty Sci Sec IV. Zoology 6:297–321.

DeBiasio RL, LaRocca GM, Post PL, Taylor DL. 1996. Myosin II transport, organization, and phosphorylation: evidence for cortical flow/solation-contraction coupling during cytokinesis and cell locomotion. Mol Biol Cell 7:1259–1282.

Field C, Li R, Oegema K 1999. Cytokinesis in eukaryotes: a mechanistic comparison. Curr Opin Cell Biol 11:68–80.

Glotzer M 1997. The mechanism and control of cytokinesis. Curr Opin Cell Biol 9:815–823.

Hales KG, Bi E, Wu JQ, Adam JC, Yu IC, Pringle JR. 1999. Cytokinesis: an emerging unified theory for eukaryotes? Curr Opin Cell Biol 11:717–725.

Julian M, Tollon Y, Lajoie-Mazenc I, Moisand A, Mazarguil H, Puget A, Wright M. 1993. Gamma-tubulin participates in the formation of the midbody during cytokinesis in mammalian cells. J Cell Sci 105:145–156.

Kaverina I, Rottner K, Small JV. 1998. Targeting, capture, and stabilization of microtubules at early focal adhesions. J Cell Biol 142: 181–190.

Kawano Y, Fukata Y, Oshiro N, Amano. M, Nakamura T, Ito M, Matsumura F, Inagaki M, Kaibuchi K. 1999. Phosphorylation of myosin-binding subunit (MBS) of myosin phosphatase by rho-kinase in vivo. J Cell Biol 29:1023–1037.

Kinoshita M, Kumar S, Mizoguchi A, Ide C, Kinoshita A, Haraguchi T, Hiraoka Y, Noda M. 1997. Nedd5, a mammalian septin, is a novel cytoskeltal component interacting with actin-based structures. Genes Dev 11:1535–1547.

Kishi K, Sasaki T, Kuroda S, Itoh T, Takai Y. 1993. Regulation of cytoplasmic division of Xenopus embryo by rho p21 and its inhibitory GDP?GTP exchange protein (rho GDI). J Cell Biol 120:1187–1195.

Kosako H, Goto H, Yanagida M, Matsuzawa K, Tomono Y, Okigaki T, Odai H, Kaibucui K, Inagaki M. 1999. Specific accumulation of Rho-associated kinase at the cleavage furrow during cytokinesis; cleavage furrow-specific phosphorylation of intermediate filament. Oncogene 18:2783–2788.

Kreis TE, Avnur Z, Schlessinger J, Geiger B. 1984. Dynamic properties of cytoskeletal proteins in focal contacts. In: Borisy GG, Cleveland DW, Murphy DB, editors. Molecular biology of the cytoskeleton. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, p 45–57.

Mabuchi I. 1986. Biochemical aspects of cytokinesis. Int Rev Cytol 101:175–212.

Mabuchi I, Ohno M. 1977. The effect of myosin antibody on the division of starfish blastomeres. J Cell Biol 74:251–263.

Mabuchi I, Hamaguchi Y, Fujimoto H, Morii N, Mishima M, Narumiya S. 1993. A rho-like protein is involved in the organization of the contractile ring in dividing sand dollar eggs. Zygote 1:325–331.

Matsumura F, Ono S, Yamakita Y, Totsukawa G, Yamashiro S. 1998. Specific localization of serine 19 phosphorylated myosin II during cell locomotion and mitosis of cultured cells. J Cell Biol 140:119–129.

Mermall V, Post PL, Mooseker MS. 1998. Unconventional myosins in cell movement, membrane traffic and signal transduction. Science 279:527–533.

Mittal B, Sanger JM, Sanger JW. 1987a. Visualization of myosin in living cells. J Cell Biol 105:1753–1760.

Mittal B, Sanger JM, Sanger JW. 1987b. Binding and distribution of filamin in permeabilized and living non-muscle and muscle cells. Cell Motil Cytoskeleton 8:345–359.

Mittal B, Sanger JM, Sanger JW. 1989. Visualization of intermediate filaments in living cells using fluorescently labeled desmin. Cell Motil Cytoskel 12:127–138.

Pedrotti B, Colombo R, Islam K. 1994. Microtubule associated protein MAP1A is an actin-binding and cross-linking protein. Cell Motil Cytoskeleton 29:110–116.

Pochapin M, Sanger JM, Sanger JW. 1983. Microinjection of Lucifer Yellow CH into sea urchin eggs and embryos. Cell Tissue Res 234:309–318.

Raich WB, Moran AN, Rothman JH, Hardin J. 1998. Cytokinesis and midzone microtubule organization in *Caenorhabditis elegans* require the kinesin-like protein ZEN–4. Mol Biol Cell 9:2037–2049.

Rappaport R. 1961. Experiments concerning the cleavage stimulus in sand dollar eggs. J Exp Zool 148:81–89.

Rappaport R. 1996. Cytokinesis in animal cells. Cambridge: Cambridge University Press, p 1–386.

Rappaport R. 1997. Cleavage furrow establishment by moving the mitotic apparatus. Dev Growth Differ 39:221–226.

Rhee D, Sanger JM, Sanger JW. 1994. The premyofibril: evidence for its role in myofibrillogenesis. Cell Motil Cytoskeleton 28:1–24.

Rieder CL, Khodjakov A, Paliulis LV, Fortier TM, Cole RW, Sluder G. 1997. Mitosis in vertebrate somatic cells with two spindles: implications for the metaphase/anaphase transition checkpoint and cleavage. Proc Natl Acad Sci USA 94:5107–5112.

Rogalski AA, Singer SJ. 1985. An integral glycoprotein associated with the membrane attachment sites of actin microfilaments. J Cell Biol 101:785–801.

Sanger JW. 1975. Changing patterns of actin localization during cell division. Proc Natl Acad Sci USA 72:1913–1916.

Sanger JW, Holtzer H. 1972. Cytochalasin-B: Effects on cytokinesis, glycogen and 3-H-D-glucose incorporation. Am J Anat 135:293–298.
Sanger JM, Sanger JW. 1980. Banding and polarity of actin filaments in interphase and cleaving cells. J Cell Biol 86:568–575.
Sanger J W, Sanger JM, Kreis TE, B. M. Jockusch BM. 1980. Reversible translocation of cytoplasmic actin into the nucleus caused by dimethyl sulfoxide. Proc Natl Acad Sci USA 77:5268–5272.
Sanger JM, Reingold AM, Sanger JW. 1984. Cell surface changes during mitosis and cytokinesis of epithelial cells. Cell Tissue Res 237:409–417.
Sanger JM, Pochapin MB, Sanger JW. 1985. Midbody sealing after cytokinesis in embryos of the sea urchin *Arbacia punctulata*. Cell Tissue Res 240:287–292.
Sanger JM, Mittal B, Pochapin M, Sanger JW. 1987. Stress fiber and cleavage furrow formation in living cells microinjected with fluorescently labeled alpha-actinin. Cell Motil Cytoskeleton 7:209–220.
Sanger JM, Mittal B, Dome JS, Sanger JW. 1989. Analysis of cell division using fluorescently labeled actin and myosin in living cells. Cell Motil Cytoskeleton 14:201–219.
Sanger J M, Dome JS, Hock RS, Mittal M, Sanger JW. 1994. Occurrence of fibers and their association with talin in the cleavage furrows of PtK2 cells. Cell Motil Cytoskeleton 27:26–40.
Sanger JM, Golla R, Safer D, Choi JK, Yu K, Sanger JW, Nachmias VT. 1995. Increasing intracellular concentrations of thymonsin b_4 in PtK2 cells: effects on stress fibers, cytokinesis and cell spreading. Cell Motil Cytoskeleton 31:307–322.
Sanger JM, Dome JS, Sanger JW. 1998. Unusual cleavage furrows in vertebrate tissue culture cells: insights into the mechanism of cytokinesis. Cell Motil Cytoskeleton 39:95–106.
Savoian MS, Earnshaw WC, Khodjakov A, Rieder CL. 1999. Cleavage furrows formed between centrosomes lacking an intervening spindle and chromosomes contain microtubule bundles, INCENP, and CHO1 but not CENP-E. Mol Biol Cell 10:297–311.
Schroeder TW. 1975. Dynamics of the contractile ring. In: Inoue S, Stephens RE, editors. Molecules and cell movement. New York: Raven Press, p 305–332.
Shu HB, Li Z, Palacios MJ, Li Q, Joshi HC. 1995. A transient association of gamma-tubulin at the midbody during the mammalian cdell division. J Cell Sci 108:2955–2962.
Shuster CB, Burgess DR. 1999. Parameters that specify the timing of cytokinesis. J Cell Biol 146:981–992.
Simon KO, Otey CA, Pavelko FM, Burridge K. 1991. Protein interactions linking actin to the plasma membrane in focal adhesions. Curr Top Membr 38:57–64.
Swan KA, Severson AF, Carter JC, Martin PR, Schnabel H, Schnabel R, Bowerman B. 1998. cyk–1: a *C. elegans* gene required for a late step in embryonic cytokinesis. J Cell Sci 111:2017–2027.
Takaishi K, Sasaki T, Kameyama T, Tsukita S, Tsukita S, Takai Y. 1995. Translocation of activated Rho from the cytoplasm to membrane ruffling area, cell-cell adhesion and cleavage furrows. Oncogene 11:39–48.
Taylor DL, Wang Y-L. 1980. Fluorescently labelled molecules as probes of the structure and function of living cells. Nature 284, 405–410.
Turnacioglu KK, Sanger JW, Sanger JM. 1998. Sites of monomeric actin incorporation in living PtK2 and REF-52 cells. Cell Motil Cytoskel 40:59–70.
Waterman-Storer CM, Sanger JW, Sanger JM. 1993. Dynamics of organelles in the mitotic apparatus of living cells: membrane and microtubule interactions. Cell Motil Cytoskeleton 26:301–312.
Wehland J, Weber K. 1981. Actin rearrangement in living cells revealed by microinjection of fluorescent phalloidin derivative. Eur J Cell Biol 24:176–183.
Wheatley SP, Wang Y-L. 1996. Midzone microtubule bundles are continuously required for cytokinesis in cultured epithelial cells. J Cell Biol 135:981–989.
Wheatley SP, Hinchcliffe EH, Glotzer M, Hyman AA, Sluder G, Wang Y-L. 1997. CDK1 inactivation regulated anaphase spindle dynamics and cytokinesis in vivo. J Cell Biol 138:385–393.
Williams BC, Riedy MF, Williams EV, Gatti M, Goldberg ML. 1995. The Drosophila kinesin-like protein KLP3A is a midbody component required for central spindle assembly and initiation of cytokinesis. J Cell Biol 129:709–723.
Yasui Y, Amano M, Inagaki N, Nagata K, Nakamura H, Saya H, Kaibuchi K, Inagaki M. 1998. Roles of Rho-associated kinase (Rho-kinase) in cytokinesis; mutations in Rho-kinase phosphorylation sites impair cytokinetic segregation of glial filaments. J Cell Biol 143:1249–1258.
Yonemura S, Nagafuchi A, Sato N. Tsukita S. 1993. Concentration of an integral membrane CD43 (leukosialin, sialophorin), in the cleavage furrow through the interaction of its cytoplasmic domain with actin-based cytoskeletons. J Cell Biol 120:437–449.
Zurek B, Sanger,J.M, Sanger JW, Jockusch BM. 1990. Differential effects of myosin antibody complexes on contractile rings and circumferential belts in epitheliod cells. J Cell Sci 97:297–306.