Signals from the Spindle Midzone Are Required for the Stimulation of Cytokinesis in Cultured Epithelial Cells

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The interaction between the mitotic spindle and the cellular cortex is thought to play a critical role in stimulating cell cleavage. However, little is understood about the nature of such interactions, particularly in tissue culture cells. We have investigated the role of the spindle midzone in signaling cytokinesis by creating a barrier in cultured epithelial cells with a blunted needle, to block signals that may emanate from this region. When the barrier was created during metaphase or early anaphase, cleavage took place only on the sides of the cortex facing the mitotic spindle. Microtubules on the cleaving side showed organization typical of that in normal dividing cells. On the noncleaving side, most microtubules passed from one side of the equator into the other without any apparent organization, and actin filaments failed to organize in the equatorial region. When the barrier was created after the first minute of anaphase, cells showed successful cytokinesis, with normal organization of microtubules and actin filaments on both sides of the barrier. Our study suggests that transient signals from the midzone of early anaphase spindles are required for equatorial contraction in cultured cells and that such signaling may involve the organization of microtubules near the equator.

INTRODUCTION

It has been known for decades that, in normal dividing cells, the cleavage furrow always appears after the onset of anaphase and between separating chromosomes (Salmon, 1989; Satterwhite and Pollard, 1992; Fishkind and Wang, 1995). Although it is widely accepted that the mitotic spindle plays a crucial role in determining the timing and location of cytokinesis (Rappaport, 1986, 1991), little is known about the source of such signals or the molecular mechanism governing the coordination between mitosis and cytokinesis.

Both the spindle pole and the spindle midzone have been implicated as a critical region for the stimulation of cortical contraction. The most compelling evidence for the role of spindle poles was provided by the micromanipulation of echinoderm embryos. In an el-

egant experiment, Rappaport (1961) generated horse-shoe-shaped cells with a spindle lying within each arm. Such cells divided not only along the plane defined by the spindle midzone, but also between adjacent poles of the two spindles, indicating that cleavage can occur independently of the spindle midzone or chromosomes. By removing different parts of the spindle, Hiramoto (1971) demonstrated that asters represent the most critical component for the stimulation of cleavage, while the rest of the spindle can be dispensed without hampering cytokinesis. To account for the stimulation of the equatorial cortex by the poles, it was further proposed that signaling is mediated by polar microtubules (Rappaport, 1986; Salmon, 1989), which emanate from spindle poles and overlap extensively near the equatorial plane. Computer simulation indicated that such a model can indeed explain cell cleavage successfully under a variety of conditions (Devore *et al.*, 1989; Harris and Gewalt, 1989).

However, there is equally convincing evidence that the spindle midzone may play a critical role in the

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stimulation of cytokinesis. For example, using micromanipulation techniques, Kawamura (1977) distorted the spindle of grasshopper neuroblasts into an arc shape, such that the midzone and the poles lay close to opposite sides of the cortex. The manipulation caused cleavage activities to increase on the side near the midzone and decrease on the side adjacent to the spindle poles, arguing in favor of the midzone as the source of cleavage signals. Rappaport and Rappaport (1974) demonstrated that, in both cultured cells and large eggs, cleavage can be induced by the spindle midzone as long as the cortex was brought close to the spindle surface. Miller *et al.* (1993) reported that when the position of cell cleavage was perturbed by the injection of calcium buffer, the pattern of furrowing appeared to be dictated by the position of the spindle interzone rather than the poles.

Although the two models of stimulation are not necessarily mutually exclusive, it is clear that additional work is required to define the interaction between the mitotic spindle and the cortex. In addition, so far most studies have been performed with early echinoderm embryos, and there are indications that the mechanism of regulation may differ substantially between these large cells and tissue culture cells (Rappaport and Rappaport, 1974). To assess the functional role of the spindle midzone in the division of cultured epithelial cells, we have created small perforations through the equator of dividing NRK cells as a physical barrier to the propagation of signals that may emanate from the spindle midzone. The approach was used previously on echinoderm embryos to demonstrate the importance of the equatorial cortex to receive the stimulation for cleavage (Rappaport and Rappaport, 1983). By using highly spread epithelial cells, we were able to control the barrier precisely relative to the location of the spindle midzone and the timing of anaphase onset. In addition, we were able to study cleavage activities in relation to the distribution of microtubules and actin filaments. Our results indicate that signals from the spindle midzone are required for the onset of cytokinesis in cultured epithelial cells, and that the coordination between mitosis and cytokinesis may involve specific organization of microtubules in the equatorial region.

MATERIALS AND METHODS

Culture and Micromanipulation of Cells

A subclone of normal rat kidney epithelial cells (NRK-52E, American Type Culture Collection, Rockville, MD) was cultured in F-12K medium (JRH Bioscience, Lenexa, KS) containing 10% fetal bovine serum (JRH), 50 μ g/ml streptomycin, and 50 U/ml penicillin. Cells were plated onto special coverslip dishes for 36–48 h (McKenna and Wang, 1989), and fed with fresh media 1 h before experiments.

Cells were maintained in a custom-built microscope stage incubator during the micromanipulation. Needles for cell perforation were prepared with a David-Kopf Model 700 vertical puller. The tip

of the needle was melted into spherical shape with a DeFonbrune microforge (Curtin Matheson Scientific, Houston, TX). Perforation of cells was achieved by slowly lowering a blunted needle onto and through the cell with a micromanipulator (Leica, Deerfield, IL). While the perforation was stable during anaphase, it disappeared rapidly during metaphase upon the removal of the needle. Therefore in experiments involving metaphase cells, the microneedle was held in place until the cell reached anaphase onset. For the perforation of anaphase cells, the needle was lifted within 4–6 s.

Immunofluorescence and Fluorescent Phalloidin Staining

For immunofluorescence of tubulin, cells were rinsed quickly with 37°C cytoskeleton buffer (containing 137 mM NaCl, 5 mM KCl, 1.1 mM Na_2HPO_4 , 0.4 mM KH_2PO_4 , 2 mM MgCl_2 , 2 mM EDTA, 5 mM piperazine-*N,N'*-bis(ethanesulfonic acid), and 5.5 mM glucose, pH 6.1; Small *et al.*, 1981), fixed with 0.5% glutaraldehyde, 0.1% Triton X-100 in cytoskeleton buffer at 37°C for 1 min, and then postfixed for an additional 10 min with 1% glutaraldehyde in cytoskeleton buffer. After rinsing twice with the cytoskeleton buffer, cells were treated with 0.1% NaBH_4 in the same buffer for 5 min to quench the autofluorescence. Nonspecific binding was blocked with phosphate-buffered saline (PBS) containing 1% bovine serum albumin (Boehringer Mannheim, Indianapolis, IN) for 10 min. Immunofluorescence staining was performed with anti- β -tubulin monoclonal antibodies (Amersham, Arlington Heights, IL) at a dilution of 1:200 at 4°C overnight. Rhodamine-conjugated secondary antibodies (Tago, Burlingame, CA) were used at a dilution of 1:100.

Cells for fluorescent phalloidin staining were fixed with 4% formaldehyde (EM Science, Gibbstown, NJ) in PBS for 10 min, followed by extraction with -20°C acetone for 5 min. Rhodamine phalloidin (Molecular Probes, Eugene, OR) was prepared by drying a 3.3- μM stock solution in methanol with nitrogen, followed by resolubilization in PBS to obtain a concentration of 220 nM. The coverslip was incubated with the phalloidin solution for 20 min at room temperature, and rinsed with PBS before observation.

Cell membrane was labeled with *N*-(5-fluorescein thiocarbonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (fluorescein-DHPE, Molecular Probes) following the procedure of Lee *et al.* (1993).

Microscopy and Image Processing

All observations were made with a Zeiss Axiovert 10 inverted microscope. Observations of phase images were performed with a 40 $\times$ /N.A. 0.65 achroplan objective lens. Immunofluorescence images were collected with a 100 $\times$ /N.A. 1.30 brightfield Neofluar objective. A 12 V, 100 W quartz-halogen lamp was used as the light source for epi-illumination.

Phase and fluorescence images were acquired with a cooled CCD camera (Star I, Photometrics, Tucson, AZ; or Princeton Instruments, Trenton, NJ). Optical sectioning was performed with a computer-controlled stepping motor at a 0.25- μm step size. Images of microtubules in the furrow were generated by adding 2 optical sections that contain focused views of microtubules in the region. To generate 3D images, slices of optical sections were first processed with a computer to remove out-of-focus fluorescence, using the nearest neighbor algorithm. Deblurred slices were then stacked and projected at different angles to generate stereo views (Fishkind and Wang, 1993). All image acquisition and processing were performed with custom-generated software. Hardcopies of images were prepared with a Sony image printer (Model UP-7100 MD).

RESULTS

Characterization of the Perforation

To assess the requirement of the spindle midzone in signaling cytokinesis, we took advantage of the spatial

resolution provided by well-spread NRK epithelial cells and imposed a small physical barrier to the communication between the spindle midzone and the cortex. The barrier was created by gently lowering a blunted microneedle onto the cell. In cells labeled with fluorescent phospholipids, the site of microneedle insertion appeared as a dark spot outlined by a rim of fluorescence (Figure 1, a and b). This suggests that the top and bottom plasma membranes had fused and sealed around the needle, forming a tunnel through the cell (hereafter referred to as perforation). In most experiments, the perforation juxtaposed the metaphase plate (or its prior location in anaphase cells). Immunofluorescence staining of tubulin confirmed that chromosomes and most associated microtubules were confined to one side of the perforation (Figure 1c), while the other side contained primarily polar microtubules.

The perforation was typically 2–4 μm in diameter under phase images. Its size appeared smaller in cells labeled with fluorescent phospholipids under fluorescence optics (Figure 1, a and b). The perforation occupied a small fraction of the space between the metaphase plate and the periphery of the cell (typically 15–20 μm in distance), thus it should block the propagation of signals from the spindle midzone while allowing the access of the cortex to other regions of the cell.

Signals from the Spindle Midzone Are Required for the Onset of Cytokinesis

When cells were perforated during metaphase or within the first minute of anaphase ($n = 9$), cleavage took place only on the side containing the mitotic spindle (Figure 2). Upon the initiation of cytokinesis, the perforation extended unidirectionally toward the mitotic spindle and changed its shape from a round hole into a slit, which then traversed across the equator with a rate much higher than that of cortical ingression during normal cleavage. The slit disappeared when it reached the periphery of the cell, where a midbody was discerned as a dark spot during late telophase (Figure 2c, arrowhead). No sign of cortical contraction was detected on the side blocked from the mitotic spindle. This partial cleavage led to the failure of cell division and creation of binucleated cells.

Different results were obtained when the perforation was created ~1.5 min or later after anaphase onset ($n = 17$). The perforation first moved and extended toward the spindle, indicating that, like cells perforated during metaphase, cortical contraction was initially limited to or biased toward the side facing the spindle (Figure 3a). However, 3–5 min after anaphase onset, the perforation started to extend bidirectionally toward both edges of the cell (Figure 3b). The cell sub-

Figure 1. Characterization of microneedle-created perforations in dividing NRK cells. The plasma membrane of an NRK cell was labeled with fluorescein-DHPE and a blunted microneedle was pressed through the cell within 1 min of anaphase onset. Upon the removal of the needle, the site of insertion remained as a phase-light region (a, arrow). Fluorescence optics shows a corresponding dark spot with a smaller apparent diameter and a bright rim (b, arrow), indicating that the needle had created a perforation through the cell. Tubulin immunofluorescence in a separate cell perforated 1.5–2 min after anaphase onset and fixed immediately shows the confinement of the spindle interzone and most interzonal microtubules to one side of the perforation (c, arrow). The opposite side of the perforation contains numerous polar microtubules. Bar, 10 μm .

sequently completed division with two cleavage furrows and midbodies (Figure 3c).

We have created perforations in different regions relative to the metaphase plate. When perforations were created during metaphase along the equator but away from the chromosomes, the outcome varied according to their relationship to the midplane of the spindle. A slight misalignment with the spindle mid-

Figure 2. Cytokinesis of an NRK cell perforated during metaphase. The perforation appeared as a round black patch (due to intensity overflow in the frame buffer) immediately upon anaphase onset and the removal of the needle (a, arrow). About 5 min after anaphase onset (b), the side of the perforation facing the mitotic spindle extended aggressively across the cell, causing the perforation to change shape into a narrow slit (arrow). Slower ingression took place at the edge of the cell unblocked by the perforation (arrowhead). No cytokinesis was observed on the side blocked by the perforation. At late telophase (c, ~12 min after anaphase onset), the slit reached the distal edge and gradually disappeared (arrow). A midbody can be discerned as a dark spot next to the edge of the cell (arrowhead). The cell became binucleated as a result of the failure in cytokinesis on one side of the perforation. The perforation appeared also to cause an uneven anaphase B, as indicated by the angular orientation of the chromosomal mass relative to the equator (b and c). Bar, 10 μ m.

plane, usually due to the rotation or movement of the spindle, would cause the perforation to disappear without affecting cytokinesis. Those remaining aligned with the midplane of the spindle showed a behavior similar to perforations placed adjacent to chromosomes as described above. In addition, irrespective of the time of generation, perforations positioned away from the equator and the spindle moved toward the center of the spindle during anaphase, while undergoing a simultaneous decrease in diameter ($n = 10$; Figure 4). Such perforations also disappeared without any detectable effects on cytoki-

nesis. These observations indicate that the signal for cortical contraction is confined to a sharply defined equatorial plane.

Organization of Actin Filaments and Microtubules in the Equatorial Region of Perforated Cells

In well spread NRK cells, actin filaments undergo an increase in organization along the equator during cytokinesis (Fishkind and Wang, 1993). When cells were perforated during metaphase or early anaphase, concentration of actin was detected only on

Figure 3. Appearance of two cleavage furrows in an NRK cell perforated during anaphase. The cell was perforated as in Figure 2, except that the perforation was performed 1.5–2 min after the onset of anaphase. Immediately after the manipulation (a), the perforation showed a slight elongation toward the center of the cell. It then elongated toward the two opposite edges of the equator, where normal ingression took place (b, ~3.5 min after anaphase onset). The cell later finished division with two cleavage furrows (c, ~6 min after anaphase onset). Phase densities corresponding to mid-bodies can be discerned in both furrows. Bar, 10 μ m.

Figure 4. Movement and disappearance of a perforation created outside the equatorial plane. The cell was perforated 1.5–2 min after anaphase onset, at a position outside the equatorial plane (a). The perforation (arrows) subsequently migrated toward the center of the spindle (asterisks) while undergoing a simultaneous decrease in diameter (b, ~1.5 min after anaphase onset; and c, ~2.5 min after anaphase onset). Finally the perforation disappeared completely without affecting the cleavage of the cell (d). Bar, 10 μ m.

the cleaving side (Figure 5a). On the other hand, cells perforated later showed similar actin organizations on both sides of the perforation (Figure 5b). Thus the organization of actin filaments mirrored the cortical cleavage activity.

The organization of anaphase microtubules in perforated cells was studied by immunofluorescence. Irrespective of the time of perforation, microtubules on the side containing the mitotic spindle showed an apparent gap of staining along the equatorial plane

(Figures 6a and 1c). Previous studies suggested that this characteristic pattern of immunofluorescence likely reflects a limited accessibility of antibodies (Sellitto and Kuriyama, 1988), due to the deposit of various proteins on microtubules. Electron microscopic observations indicated that microtubules from the two half-spindles overlap extensively along the equator, with their ends penetrating for a limited distance (up to 5 μ m, average = ~1–2 μ m) into the opposite half-spindles (Mastronarde, *et al.*, 1993).

Figure 5. Organization of actin filaments in perforated cells. Cells were fixed at mid-cytokinesis and stained with rhodamine phalloidin. Images are shown in pseudocolor, with red color corresponding to highest intensity of fluorescence, followed by yellow, green, and blue. When cells were perforated during metaphase (a), actin filaments became concentrated only on the side containing the mitotic spindle (arrow). Cells perforated after the first minute of anaphase show the concentration of actin filaments on both sides of the perforation (b, arrows). Bar, 10 μ m.

Figure 6. Organization of microtubules in perforated cells. The site of perforation is indicated by asterisks. (a) The typical pattern of microtubules in the spindle interzone. A cell was perforated 1.5–2 min and fixed ~3 min after anaphase onset. A gap of staining is apparent in the spindle interzone along the equator. When the cell was perforated during metaphase and fixed ~5 min after anaphase onset (b), microtubules on the noncleaving side of the perforation appear as a network extending far beyond the equatorial plane. Most microtubules on the spindle side are out of focus in this image. A stereo pair (e) shows microtubules in a cell similarly perforated during metaphase and fixed during mid-cytokinesis. The difference in microtubule organization between the two sides of the perforation is striking. When the cell was perforated 1.5–2 min and fixed ~5 min after anaphase onset (c), most microtubules were found to terminate near the equator on the side without the spindle. However, the apparent gap of staining was not well developed at this point. When a similarly perforated cell was fixed during telophase (d, ~7 min after anaphase onset), similar organization of microtubules, with an apparent gap of staining, was observed on either side of the perforation. Bar, 10 μ m.

On the other side of the perforation without the mitotic spindle, the pattern of microtubule staining varied dramatically with the timing of perforation. In

cells perforated at metaphase or early anaphase ($n = 7$), microtubules appeared as a network reminiscent of those in interphase cells (Figure 6, b and e). Many

microtubules can be traced from one side of the equator far into the other side as soon as 5 min after anaphase onset. On the other hand, in cells perforated after the first 1.5 min of anaphase, most microtubules appeared to terminate near the equator during early anaphase, without showing a well-defined gap of staining (Figure 6c; $n = 15$). The gap at the equator became clear subsequently and, at telophase, both sides of the perforation showed a similar appearance of microtubule staining (Figure 6d, $n = 10$).

DISCUSSION

The method of cell perforation has been applied in the past to probe the signaling of cytokinesis (Rappaport and Rappaport, 1974; Rappaport and Rappaport, 1983). However, these previous studies were performed exclusively with echinoderm embryos and were limited by the difficulty in detecting chromosomes, anaphase onset, and cytoskeletal structures. In the present study, we have applied this method to well spread cultured epithelial cells, which allowed us to assess more specifically the requirement of signals that may emanate from the spindle midzone at early anaphase. In addition, the superior optical quality of the system allowed us to obtain important information on the relationship between cleavage activities and the organization of actin filaments and microtubules in cultured cells.

Role of Spindle Midzone in Stimulating Cytokinesis

Previous studies provided strong evidence that the integrity of the mitotic spindle is required transiently for the stimulation of cytokinesis (Mullins and Snyder, 1981; Rappaport, 1986). When the position of the mitotic spindle is shifted by micromanipulation, the plane of cell cleavage moves accordingly (Rappaport, 1985). However, once furrowing is established, the spindle can be destroyed without inhibiting cleavage (Hamaguchi, 1975; Mullins and Snyder, 1981).

In the present study, we showed that cortical cleavage in cultured cells can be inhibited by imposing a small perforation immediately adjacent to the region occupied by metaphase chromosomes. While the perforation is expected to serve as a barrier to signals that originate from the spindle midzone, its small size relative to the spread area of the cell suggests that the cortex most likely had maintained the access to other regions of the cell such as the spindle poles. Moreover, there was no apparent reduction in the density of microtubules in the vicinity of the equatorial cortex. Our results thus indicate that, at least for cultured epithelial cells, signals from the spindle midzone are required for the stimulation of cytokinesis. Since cytokinesis was inhibited only when cells were perforated before the first minute of anaphase, it is likely

that the signal is released at anaphase onset and transmitted toward the cortex during the first minute of anaphase. Once the signal reaches the cortex, cleavage can proceed to completion without further stimulation from the spindle midzone. Such a mechanism, based on signals originating from the region of separating chromosomes, would ensure that cytokinesis occurs after the separation of chromosomes and in the region between separating chromosomes.

From the strong dependence of the outcome on the location of the perforation, as well as the drastic change in the shape of the perforation into a slit, the stimulation for cytokinesis is probably highly localized along the equatorial plane of cultured cells. This, in addition to the effectiveness of a relatively small perforation in blocking cleavage, argues against a mechanism mediated by freely diffusible molecules. Possibly the signal migrates along an equatorially localized structure or involves localized mechanical interactions. It is also clear that the spindle signal lies upstream from the reorganization of both microtubules and cortical actin filaments, as indicated by the dramatic differences in the staining pattern between the two sides of the perforation created during metaphase.

Possible Role of Microtubules in Cytokinesis

It has been known for many years that microtubules undergo a dramatic elongation upon anaphase onset (Salmon, 1989). However, most microtubules overlap extensively along the equator and penetrate for a limited distance into the opposite half-spindle (Mastronarde *et al.*, 1993). Our results suggest that signals released from the region of separating chromosomes are required for such an arrangement of microtubules. In addition, since microtubules can exert positive effects on cortical dynamics and actin structure (Fishkind, Silverman, and Wang, unpublished data), the organization of microtubules in the equatorial region may then define the site of cortical contractility through, possibly, the accumulation of plus-end-directed motors, associated molecules, or organelles. It is interesting to note that a number of microtubule-associated proteins, including motor proteins, have been found to dissociate from chromosomes during anaphase onset and to accumulate in the equatorial zone before the onset of cytokinesis (Margolis and Andreassen, 1993). These proteins likely account for the shielding of microtubules from immunofluorescence staining along the equator, and also represent attractive candidates for regulating the elongation of microtubules and for mediating the transmission of signals to the cortex.

Our conclusion thus differs from those established previously with early embryos, which indicate that a pair of appropriately positioned spindle poles is suf-

ficient for the stimulation of cytokinesis (Rappaport, 1986), and that cleavage-like activities can occur in regions far away from chromosomes under a number of conditions (Hird and White, 1993; Rappaport and Rappaport, 1994). One possibility is that tissue culture cells differ from embryos in the regulation of cytokinesis. Due to the sheer size of embryos and the long distance between chromosomes and the cortex, it is possible that early embryonic cells use alternative or additional means to facilitate the communication between the spindle and the cortex.

Do spindle poles and polar microtubules play any role in the cytokinesis of cultured cells? Our results do not rule out the possibility that spindle poles may play an assisting role, such as inducing local relaxation of the cortex (White and Borisy, 1983; Bray and White, 1988). Additionally, the results with cells perforated during anaphase (Figure 3) suggest that polar microtubules may share the same ability as interzonal microtubules in mediating cytokinesis, as long as they receive proper signals to establish a specific organization. Thus while spindle midzone signals may be required for establishing the organization of microtubules along the equator, additional planes of cleavage could be created as long as the plus ends of microtubules and associated proteins happen to organize in a particular fashion. This may explain why the source of stimulation appears to shift among different parts of the spindle, according to the cell type and to the geometric relationship between the spindle and the cortex (Rappaport and Rappaport, 1974). A systematic investigation of the organization of microtubules in relation to cortical activities under various conditions should yield additional important clues to the mechanism of signaling for cytokinesis.

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