

# Inhibition of Chromosomal Separation Provides Insights into Cleavage Furrow Stimulation in Cultured Epithelial Cells

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Submitted November 4, 1997; Accepted May 18, 1998  
Monitoring Editor: Paul Matsudaira

While astral microtubules are believed to be primarily responsible for the stimulation of cytokinesis in *Echinoderm* embryos, it has been suggested that a signal emanating from the chromosomal region and mediated by the interzonal microtubules stimulates cytokinesis in cultured mammalian cells. To test this hypothesis, we examined cytokinesis in normal rat kidney cells treated with an inhibitor of topoisomerase II, (+)-1,2-bis(3,5-dioxopiperaz-inyl-1-yl)propane, which prevents the separation of sister chromatids and the formation of a spindle interzone. The majority of treated cells showed various degrees of abnormality in cytokinesis. Furrows frequently deviated from the equatorial plane, twisting daughter cells into irregular shapes. Some cells developed furrows in regions outside the equator or far away from the spindle. In addition, F-actin and myosin II accumulated at the lateral ingressing margins but did not form a continuous band along the equator as in control cells. Imaging of microinjected 5- (and 6-) carboxymethylrhodamine-tubulin revealed that a unique set of microtubules projected out from the chromosomal vicinity upon anaphase onset. These microtubules emanated toward the lateral cortex, where they delineated sites of microtubule bundle formation, cortical ingression, and F-actin and myosin II accumulation. As centrosome integrity and astral microtubules appeared unperturbed by (+)-1,2-bis(3,5-dioxopiperaz-inyl-1-yl)propane treatment, the present observations cannot be easily explained by the conventional model involving astral microtubules. We suggest that in cultured epithelial cells the organization of the chromosomes dictates the organization of midzone microtubules, which in turn determines and maintains the cleavage activity.

## INTRODUCTION

To ensure faithful transmission of the genome from one generation to the next, mitosis and cytokinesis are coordinated temporally and spatially. Thus, only after the completion of mitosis does the cleavage furrow bisect the cell. Furthermore, in most cases the cleavage furrow forms equidistant between the spindle poles, coincident with the prior location of the metaphase plate.

In *Echinoderm* embryos the location of centrosomes appears to dictate the plane of cleavage, as indicated by elegant micromanipulation experiments (Rappaport, 1961, 1985, 1991a; Salmon and Wolniak, 1990). These observations are consistent with the hypothesis that microtubules emanating from spindle poles are sufficient for the stimulation of cytokinesis (White and Borisy, 1983; Devore *et al.*, 1989; Harris and Gewalt, 1989; Oegema and Mitchison, 1997), even though the nucleus may play an enhancing role in the process (Rappaport, 1991b). While it is often assumed that the same principle should apply to other types of animal cells, recent studies suggest that there may be substantial complexities and variations (Oegema and Mitchi-

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son, 1997). For example, a number of chromosome-associated proteins have been implicated in cytokinesis, based on their relocation to the equatorial cortex during telophase (Cooke *et al.*, 1987; Andreassen *et al.*, 1991; Martineau *et al.*, 1995; Eckley *et al.*, 1997). In addition, cleavage can be blocked by imposing a physical barrier between the cortex and the spindle midzone (Cao and Wang, 1996), suggesting that certain components from the spindle midzone are essential. Our recent results further indicate that in cells with multipolar spindles, cytokinesis is closely correlated with the organization of midzone microtubule bundles and associated proteins rather than the position of spindle poles (Wheatley and Wang, 1996).

To address the role of separating chromosomes and midzone microtubules in cytokinesis, it would be informative to disrupt chromosomal separation and observe the effects on microtubules and cytokinesis. One approach is to inhibit the activity of topoisomerase II (topo II)<sup>1</sup> with a drug, which in mammalian cells prevents the separation of chromosomal arms while allowing centromeres to separate for a limited distance (Gorbsky, 1994; Sumner, 1995). Similar effects have been observed in flies (Buchenau *et al.*, 1993) and genetically disrupted yeast (Holm *et al.*, 1985; Uemura and Yanagida, 1986). Interestingly, cytokinesis proceeds but the chromatin becomes randomly partitioned into the daughter cells (Gorbsky, 1994). Although the degree of disruption to cytokinesis is unclear, in the presence of incompletely segregated chromosomes one might expect severe constraints to the organization of midzone microtubules and disruptions to events that might rely specifically on this set of microtubules.

In this study we treated normal rat kidney (NRK) epithelial cells with a topo II inhibitor, (+)-1,2-bis(3,5-dioxopiperazinyl-1-yl)propane (ICRF-187). Our results indicate that while cytokinesis is attempted on schedule, cleavage furrows in most cells deviated from their normal positions causing gross distortions in the pattern of cleavage. Furthermore, we discovered that the initiation sites of such distorted cleavage are defined by a set of microtubules that emanates laterally from the region of tangled chromosomes toward the cell cortex during early anaphase. This set of microtubules appears to be involved in the formation of midzone microtubule bundles and the concentration/contraction of cortical F-actin and myosin II.

## MATERIALS AND METHODS

Unless otherwise stated, materials were obtained from Sigma Chemical (St. Louis, MO).

<sup>1</sup> Abbreviations used: ICRF-187, [(+)-1,2-bis(3,5-dioxopiperazinyl-1-yl)propane]; NRK, normal rat kidney; TAMRA, 5-(and-6)-carboxytetramethylrhodamine; TD60, telophase disk antigen of 60 kDa; topo II, topoisomerase II.

## Cell Culture

A well-spread subclone of Normal Rat Kidney epithelial cells (NRK-52E; American Type Culture Collection, Rockville, MD) was cultured in Kaighn's modified F12 medium supplemented with 10% FBS (JRH Biosciences, Lenexa, KS), 50 U/ml penicillin, and 50 µg/ml streptomycin, on glass chamber dishes as described by McKenna and Wang (1989).

## Drug Treatments

A 10 mg/ml stock solution of ICRF-187 (Pharmacia and Upjohn Inc., Albuquerque, NM; manufactured as Zinecard, sometimes referred to as ADR-529 or dexrazoxane) was prepared in 0.2 N HCl and stored at -20°C. ICRF-187 was diluted to a final concentration of 20 µg/ml in prewarmed F12K (complete) medium.

Amsacrine and nocodazole were diluted into complete F12K medium from 500× stock solutions in DMSO kept at -20°C. Amsacrine was used at a final concentration of 8.6 µg/ml, and nocodazole was used at 2.5 µg/ml. Cytochalasin D was stored as a stock solution of 2 mM in DMSO and was diluted into the F12K medium to obtain a final concentration of 1 µM.

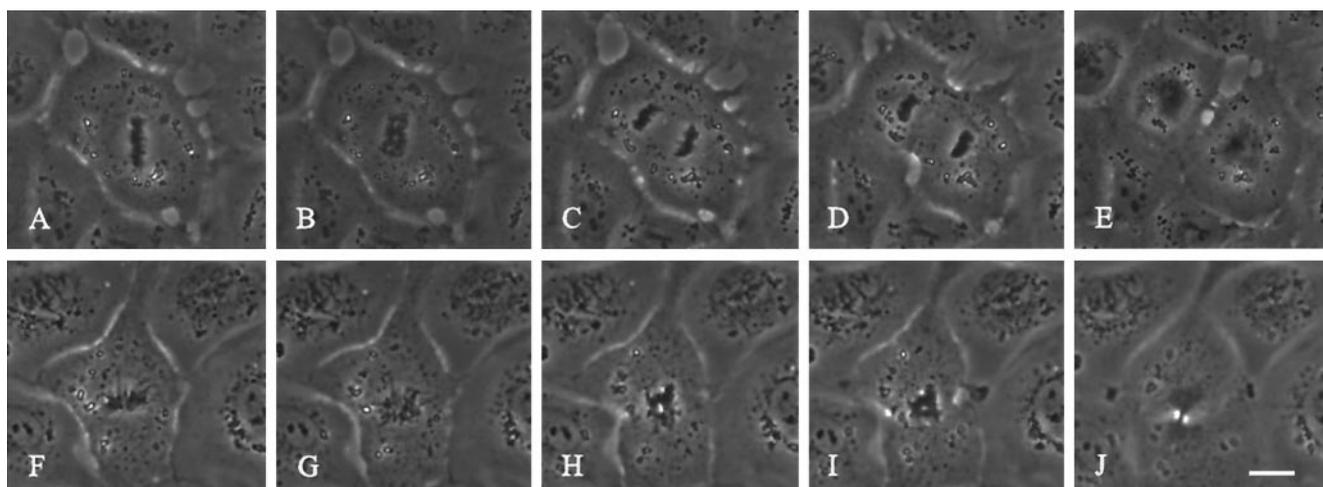
## Fixation and (Immuno)fluorescence Staining

To preserve microtubules, cells were fixed using glutaraldehyde (Polysciences, Warrington, PA) as described by Wheatley and Wang (1996). To simultaneously preserve microtubules and telophase disk antigen of 60 kDa (TD60), cells were fixed in 95% chilled (-20°C) methanol containing 5 mM EGTA (pH 6.0) for 10 min. For pericentrin localization, cells were fixed with a mixture of formaldehyde (EM Sciences, Gibbstown, NJ) and Triton X-100, as detailed by Wheatley and Wang (1996).

Cells were fixed for myosin II staining using a combination of glutaraldehyde and formaldehyde as follows. Cells were rinsed in prewarmed PBS, fixed for 1 min with 0.1% glutaraldehyde-1% formaldehyde containing 0.3% Triton X-100 prepared in warm Cytoskeleton Buffer [CB; 137 mM NaCl, 5 mM KCl, 1.1 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.4 mM KH<sub>2</sub>PO<sub>4</sub>, 2 mM MgCl<sub>2</sub>, 2 mM EGTA, 5 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid), 5.5 mM glucose, pH 6.1; Small, 1981]. They were then rinsed in CB and postfixed for 15 min with 0.5% glutaraldehyde in CB. After rinsing in CB, samples were treated for 5 min with 0.5 mg/ml NaBH<sub>4</sub>, rinsed again with CB, and then with PBS before staining was begun.

Before probing, all cells were incubated in PBS/BSA (PBS containing 1% BSA; fraction V; Boehringer Mannheim, Indianapolis, IN) for 1 h at room temperature. PBS/BSA was used as the diluent for all antibodies. Diluted antibodies were centrifuged at room temperature for 15 min at 13,000 × g before use. Between antibody applications, samples were washed with PBS and incubated in PBS/BSA for at least 30 min at room temperature. We probed myosin II with anti-platelet myosin II (a gift from K. Fujiwara, National Cardiovascular Center, Osaka, Japan) and FITC-conjugated anti-rabbit IgG (Tago, Burlingame, CA), both diluted by 1/50. Pericentrin was localized using affinity-purified rabbit anti-pericentrin antibodies (a gift from S. Doxsey, University of Massachusetts Medical School) at a 1:1000 dilution, and FITC-conjugated anti-rabbit secondary antibodies (Tago, as above). TD60 was localized using 1/200 JH antiserum (provided by D. Palmer, University of Washington, Seattle, WA) and 1/50 FITC-conjugated anti-human IgG secondary antibodies (Sigma).

To counterstain for F-actin, cells were incubated for 30 min at room temperature with 200 nM FITC-phalloidin or TRITC-phalloidin (Molecular Probes, Eugene, OR) in PBS. To visualize the chromosomes, cells were incubated for 15 min at room temperature with 10 µg/ml Hoechst 33258, diluted in PBS from a 10 mg/ml stock in DMSO.



**Figure 1.** Phase images of NRK cells treated with the topo II inhibitor, ICRF-187. Control (A–E) or ICRF-187-treated cells (F–J) were monitored as they progressed from metaphase (A and F) through anaphase onset (B and G), anaphase (C and H), telophase (D and I) and cytokinesis (E and J). In ICRF-187-treated cells, sister chromatids failed to separate. The cleavage of this cell appeared relatively normal and resulted in apparently even partitioning of the chromatin between the two daughter cells (J). Bar, 20  $\mu\text{m}$ .

#### *Preparation and Microinjection of 5- (and 6-) Carboxytetramethylrhodamine (TAMRA)-Tubulin*

Tubulin was prepared according to Williams and Lee (1982) and reacted with succinimidyl ester (TAMRA-SE; Molecular Probes), as described by Sammak and Borisy (1988). The molar ratio of conjugated rhodamine to tubulin dimer was 1.2. TAMRA-labeled tubulin was injected as described previously by Wheatley and Wang (1996).

#### *Microscopy and Data Collection*

Cells were viewed using an Axiovert 10 microscope (Carl Zeiss, Thornwood, NY) with a 40 $\times$ , NA 0.75 plan-achroplan phase contrast lens or a 100 $\times$ , NA 1.3 Neofluar lens. Fluorescence was detected using epifluorescent optics, and images were detected using a cooled charge coupled device camera (TE/CCD-576EM; Princeton Instruments, Trenton, NJ). All images were processed by subtraction of camera dark noise and archived using custom designed software. To construct microtubule organization, cells microinjected with TAMRA-tubulin were fixed, and optical sectioning was performed using a computer-controlled stepping motor at 0.25  $\mu\text{m}$  step size. The images were deconvolved with the nearest neighbor algorithm as described by Wang (1998). Recognizable segments of microtubules were traced in each deconvolved optical section using Corel Draw (Corel, Ottawa, Ontario, Canada). The stack of traced segments was then reconstructed into perspective views at various angles using custom software. Hard copies were prepared with a Kodak Color Ease Printer (Eastman Kodak, Rochester, NY).

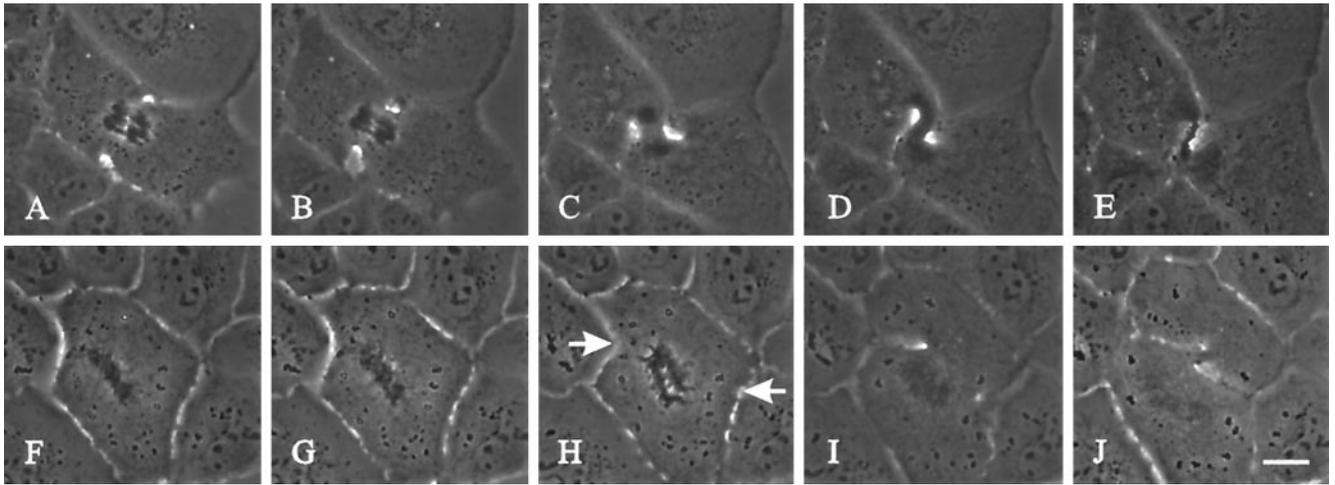
## RESULTS

Cells treated with 20  $\mu\text{g}/\text{ml}$  ICRF-187 entered and exited mitosis normally, as there was no detectable delay in anaphase onset, chromosome decondensation, or nuclear envelope reformation. This is consistent with a previous report indicating that a related compound, ICRF-193, has no detectable effect on the progression of cell cycle (Ishida *et al.*, 1994). However, sister chromosome arms failed to separate despite the

normal anaphase onset, resulting in a tangled mass of chromosomes at the cell center (compare Figure 1, B–D, with Figure 1, G–I and Figure 2). In addition, the physical constraint apparently led to the inhibition of spindle pole-to-pole separation in anaphase B. Effects of the treatment were evident 15–20 min after exposure to the drug and lasted for more than 8 h. As a positive control, cells were treated with amsacrine (8.3  $\mu\text{g}/\text{ml}$ ), a topo II inhibitor with a structure distinct from ICRF-187 (Sumner, 1995). Amsacrine caused responses similar to ICRF-187 during division; however, the mitotic index of the population decreased after exposure for 1 h, suggesting that its effect was not limited to anaphase.

#### *ICRF-187-Treated Cells Divide Abnormally*

ICRF-187 had no detectable effect on the timing of cytokinesis onset. A fraction (37%) of cells appeared to divide normally, randomly partitioning the tangled chromosomes between the two daughter cells (Figure 1, F–J). However, abnormal cytokinesis (Figure 2) was observed in the majority of treated cells (63%; 17 of 27 cells). In 30% of treated cells, ingression appeared to initiate normally but later deviated from the equatorial plane. The two lateral edges followed different paths, causing elongation and distortion of the cleavage furrow (Figure 2, C–E). In 22% of ICRF-187-treated cells, furrows initiated away from the equatorial plane as defined by the metaphase plate (Figure 2H, arrows), partitioning all the chromosomes into one daughter cell (Figure 2J) or forming ectopic furrows far away from the spindle (Figure 3, arrows; see



**Figure 2.** Abnormal cleavage in topo II-inhibited cells. In some cells, furrows initiated along the equatorial plane (A and B), but veered off subsequently (C and D) causing gross distortion of the cleavage furrow (D and E). In other cells, ingression initiated away from the equatorial plane as defined by the metaphase plate (H, arrows), causing all the chromatin to partition into one of the daughter cells (H and I). Bar, 20  $\mu\text{m}$ .

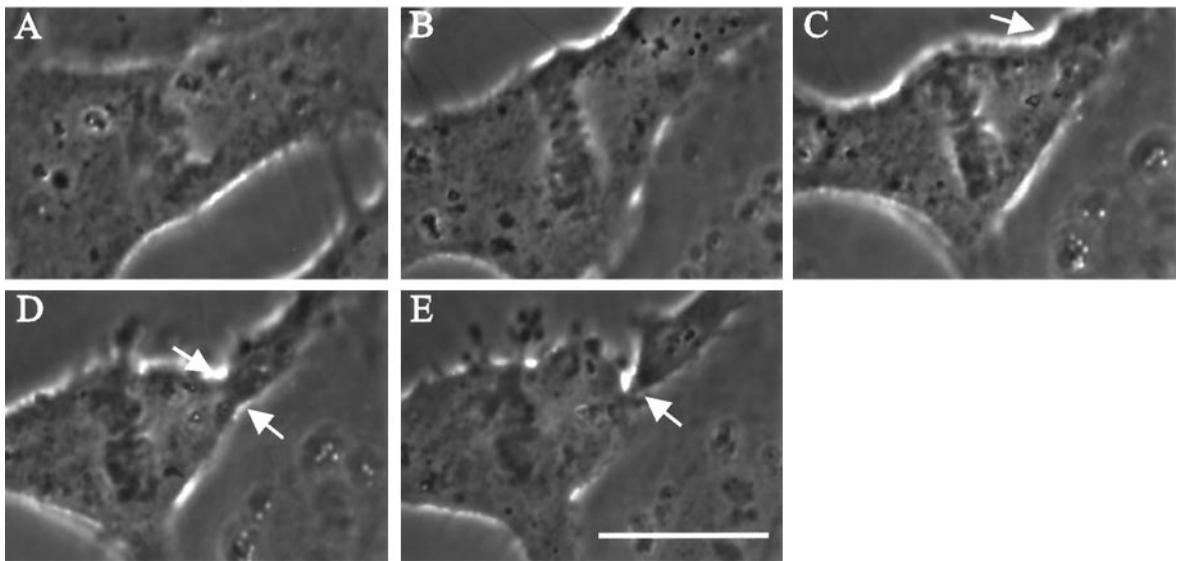
also Figure 7C). In 15% of treated cells, furrows regressed during various stages of cytokinesis.

Further differences were noticed upon examination of the organization of F-actin and myosin II. Untreated, well-spread NRK epithelial cells typically showed concentrations of F-actin and myosin II along the equator (Figure 4, A and B). By contrast, in ICRF-187-treated cells, F-actin and myosin II were concentrated only at lateral margins of the cortex where ingression took place (Figure 4, D and E; confirmed by

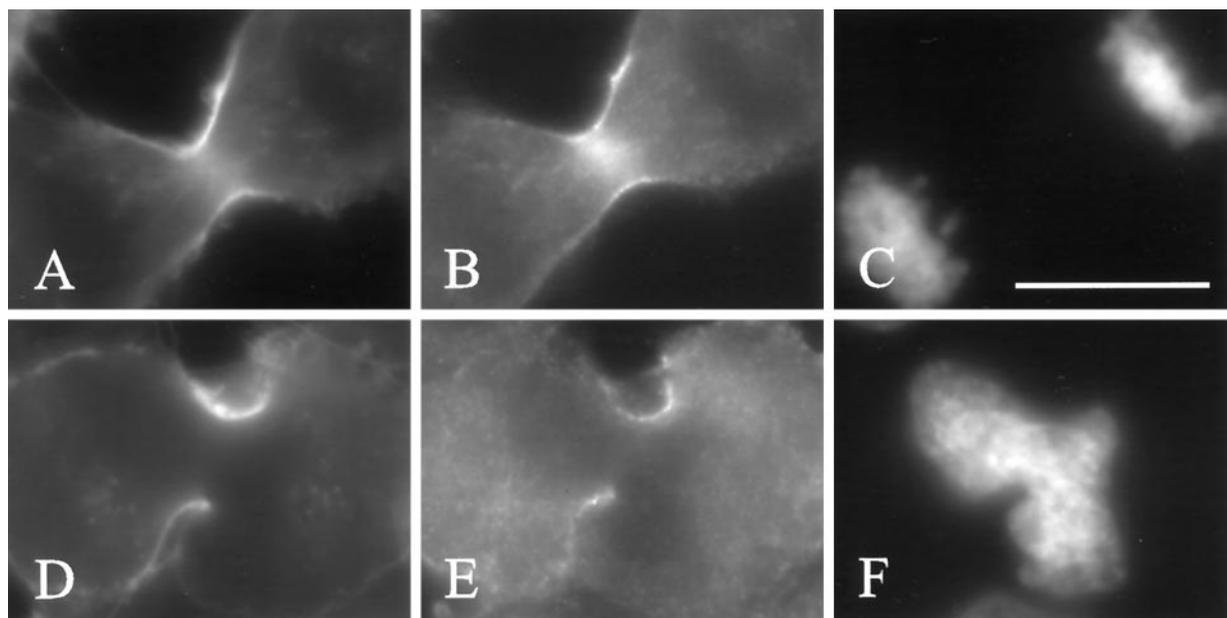
our unpublished observations with three-dimensional optical sections and reconstruction).

***Microtubules Emanate from Tangled Chromosomes toward the Lateral Cortex in ICRF-187-treated Cells***

We first examined microtubule organization by microinjection of TAMRA-tubulin and time-lapsed fluorescence imaging (Figure 5). In control cells, a new set of microtubules, the midzone microtubules, appeared



**Figure 3.** Formation of ectopic furrows in ICRF-187-treated cells. While furrows in topo II-inhibited cells usually initiated near the equator, they were occasionally found far away from the tangled chromosomes (C–E, arrows), creating a small cytoplasm near one end of the cell. Bar, 10  $\mu\text{m}$ .



**Figure 4.** Localization of F-actin and myosin II in control (A–C) and ICRF-187-treated (D–F) cells. Fixed cells were probed for myosin II (B and E) and counterstained with FITC-phalloidin to localize F-actin (A and D), and with Hoechst 33258 for the chromatin (C and F). In control cells, both myosin II and F-actin were present as a continuous band across the equatorial cortex (A and B). In topo II-inhibited cells, F-actin and myosin II were concentrated only at the lateral margins of the cell (D and E). Distortion of the cleavage furrow is also evident in this cell. Bar, 20  $\mu\text{m}$ .

between the separating chromosomes during anaphase and became bundled before the onset of cleavage (Figure 5, B–D). ICRF-187-treated cells constructed a normal bipolar metaphase spindle as expected (Figure 5E; see also Gorbsky, 1994). During anaphase, many aspects of microtubule organization appeared normal: kinetochore microtubules shortened (Figure 5, F–H), astral microtubules elongated (Figure 5, F–J, and Figure 6), and spindle poles remained intact as indicated by pericentrin localization (our unpublished observations). However, due to the inhibition of sister chromatid separation, pole-to-pole separation was inhibited and midzone microtubule bundles never appeared between the two sets of kinetochore microtubules (compare Figure 5, G–H, with Figure 5, B and C).

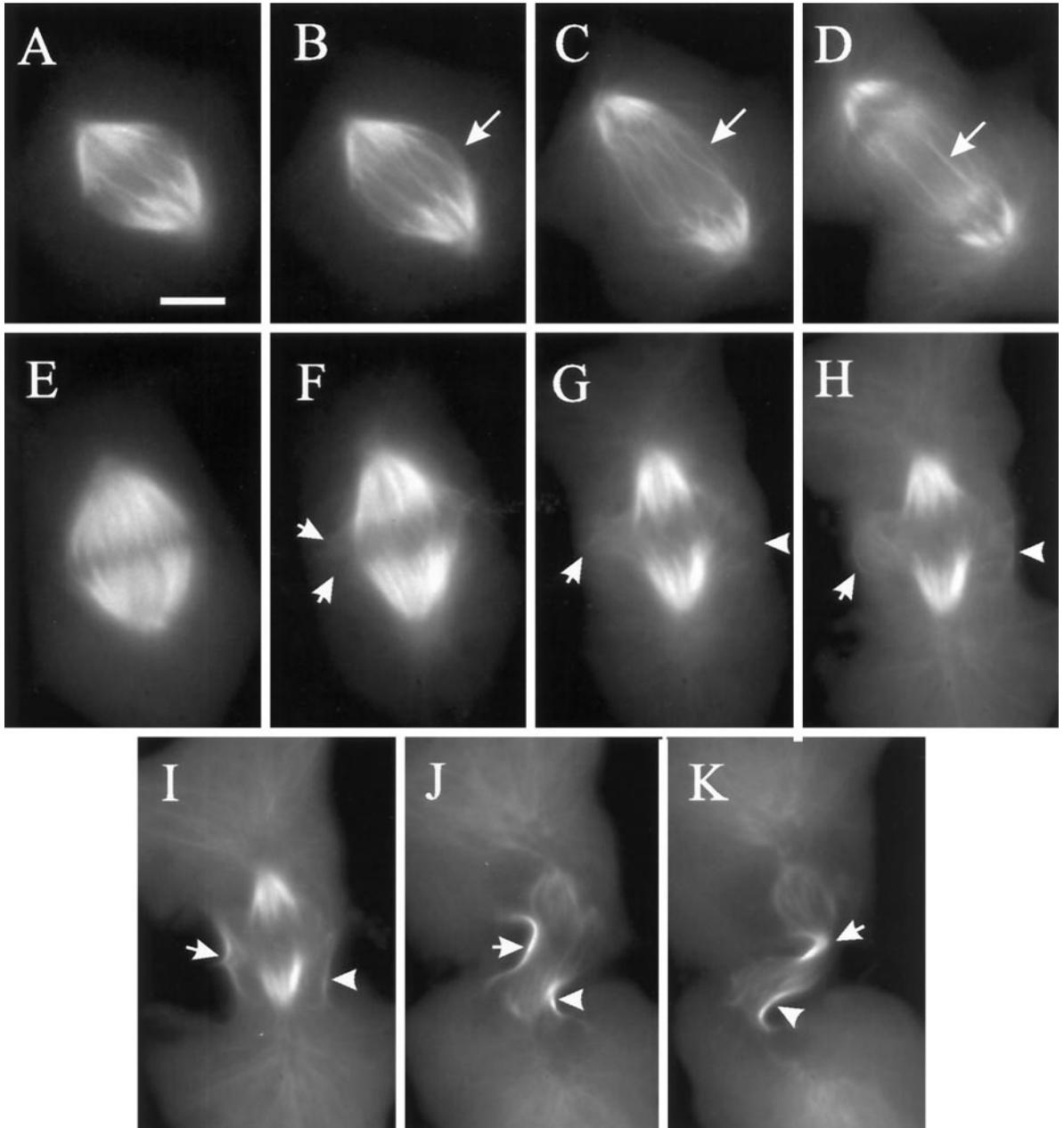
The most novel aspect of ICRF-187-treated cells was the extension of a discrete set of microtubules from the region occupied by tangled chromosomes toward lateral margins of the cortex, during the first 2 min of anaphase (Figure 5, F–H, arrows and arrowheads). These microtubules initially appeared as diffuse fluorescence “puffs.” Large microtubule bundles and cortical ingression subsequently developed (Figure 5, I–K), exactly where these microtubule puffs reached the lateral cortex (Figure 5G, arrow and arrowhead).

More detailed organization of microtubules was obtained when injected cells were fixed and examined with optical sectioning and deconvolution. In images

of reconstructed microtubules (Figure 6), microtubules in the puff were found to originate near the central region of the spindle and extend along a direction almost perpendicular to the spindle axis, without an apparent continuity with polar or kinetochore microtubules. These microtubules appear to merge with polar microtubules at their distal ends, suggesting that cortical microtubule bundles consist of both puff and polar microtubules.

#### *Cortical Microtubule Bundles Delineate the Site of the Cleavage*

We noticed that the site of ingression, including ectopic furrows, was always delineated by a bright bundle of microtubules (Figure 5, I–K, arrows and arrowheads; Figure 7, A–C). These microtubule bundles remained perfectly localized at the site of ingression, even when the furrow deviated away from the equatorial plane (Figure 5, I–K), and appeared to be the counterpart of midzone microtubule bundles implicated in the regulation/maintenance of cleavage activities in untreated cells (Wheatley and Wang, 1996). However, while they spanned the interchromosomal area in control cells (Figure 5, B–D), in most ICRF-187-treated cells these microtubule bundles were localized only near ingressing lateral margins (Figure 5, I–K, and Figure 7, A and B, arrows). Their localization

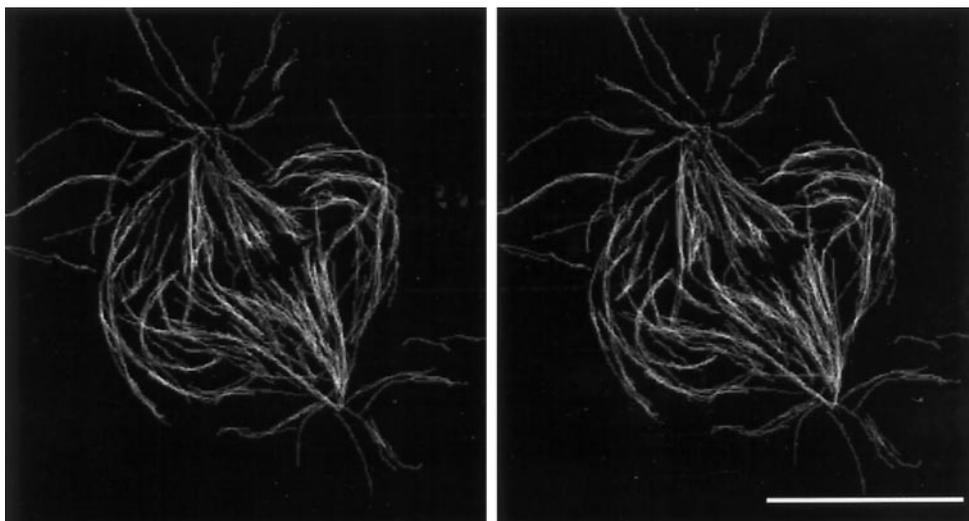


**Figure 5.** Aberrant interzonal microtubule organization in topo II-inhibited NRK cells. Prometaphase cells were injected with TAMRA-tubulin and monitored by time lapse fluorescence microscopy. As control cells entered anaphase (B), an array of microtubule bundles along the spindle axis developed in the spindle interzone (B–D, arrows). Concomitantly, spindle poles moved apart (C and D). In topo II-inhibited cells, as chromosomes attempted to separate, microtubules began to emanate from the chromosomal region (F, arrows). These microtubules puffed out toward the lateral margins of the cell (G and H, arrows and arrowheads), where cortical microtubule bundles and the cleavage furrow developed subsequently (I–K). These bundles remained localized at the ingressing furrow (I–K). The furrow on the right margin initiated below the equator, while the furrow on the left margin veered above the equatorial plane. Astral microtubules elongated extensively during anaphase and telophase, filling the distal expanses of the cell with faint linear structures (H–K). Pole-to-pole distance remained relatively constant in topo II-inhibited cells. Bar, 10  $\mu\text{m}$ .

paralleled the lateral concentration of F-actin and myosin II described above (Figure 4, D and E).

To elucidate the causal relationship of microtubule puffs, bundles, and cleavage, ICRF-187-treated cells

were injected with rhodamine-labeled tubulin and treated with cytochalasin D at anaphase onset to prevent furrow formation (Figure 8). Like control cells, cytochalasin-treated cells developed lateral puffs of



**Figure 6.** Three-dimensional organization of microtubules in the puff. A cell injected with TAMRA-tubulin was fixed at a stage similar to that shown in Figure 5H. The cell was optically sectioned and deconvolved, and microtubules visible in each section were traced using Corel Draw. The traces were then reconstructed into a stereo pair. Many microtubules in the puff originate near the central region of the spindle and project toward the cortex. They appear to be independent of astral or kinetochore microtubules but merge with astral microtubules near the lateral margins. Bar, 10  $\mu\text{m}$ .

microtubules shortly after anaphase onset (Figure 8A). Cortical microtubule bundles subsequently formed despite the absence of a cleavage furrow (Figure 8, A' and B). In addition, upon the removal of cytochalasin D, cytokinesis initiated where the microtubule bundles were located (Figure 8B'). Bundles often became more intense as the furrow progressed, possibly due to the addition of cytoplasmic microtubules caught up by the progressing furrow.

Additional evidence for the requirement of microtubule bundles in cytokinesis came from cells treated with nocodazole. Midzone microtubule bundles became increasingly resistant to nocodazole-induced depolymerization at late anaphase (Wheatley and Wang, 1996). A cleavage furrow formed and persisted only when the associated microtubule bundles remained visible (Figure 7, E–G). Detours in cleavage and distortions of the furrow were also observed in these cells, similar to ICRF-187-treated cells (Figure 7, E–G, arrows). The ingression continued along a twisted path for a prolonged period of time while the associated microtubule bundle diminished gradually.

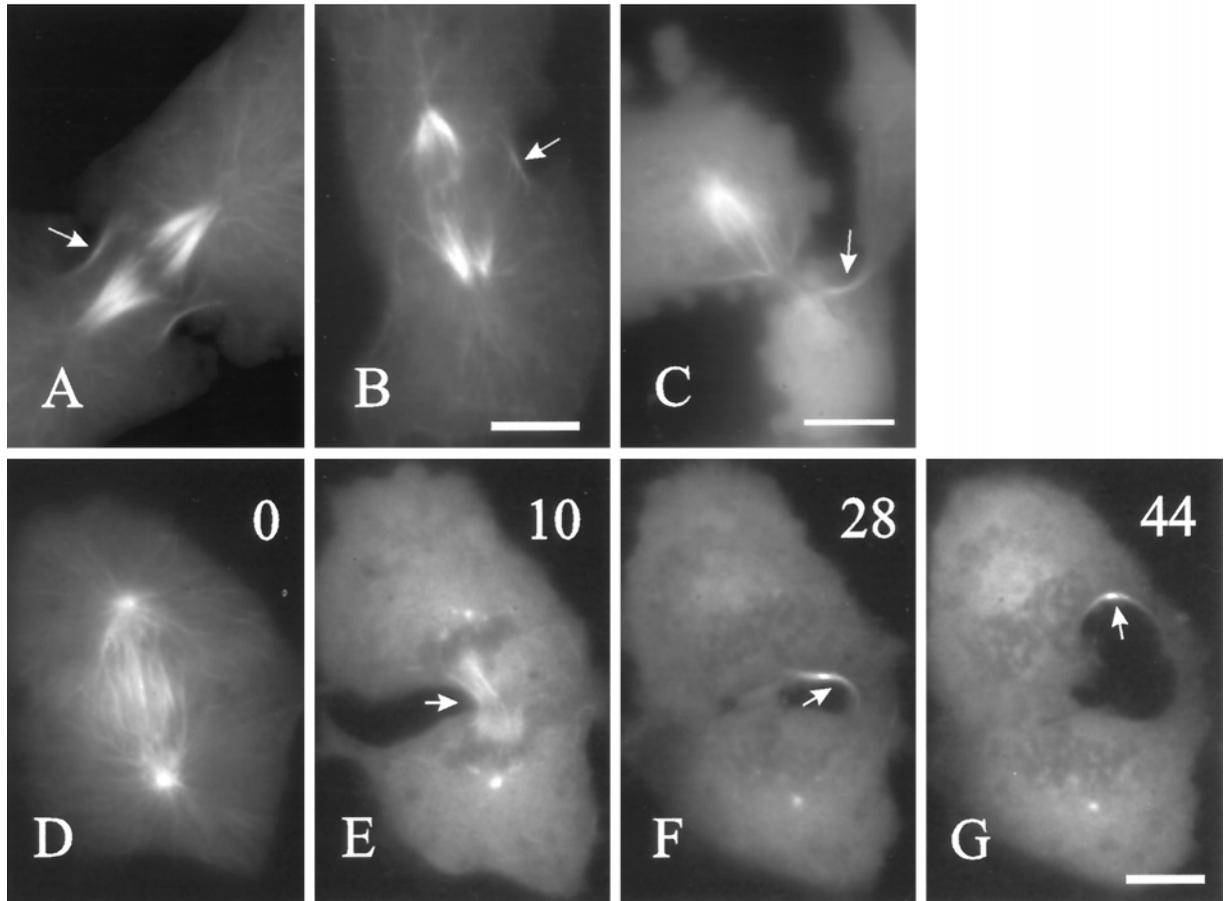
## DISCUSSION

Despite many years' investigation, it remains unclear how cytokinesis is coordinated spatially and temporally with the separation of chromosomes. Although it is agreed that the position of the mitotic apparatus somehow dictates the plane of cleavage (see reviews by Mabuchi, 1986; Salmon, 1989; Rappaport, 1991a; Satterwhite and Pollard, 1992; Fishkind and Wang,

1995; Oegema and Mitchison, 1997; Glotzer, 1997), the contribution of each component of the mitotic apparatus in signaling cytokinesis is an area of lively controversy.

A long-held view maintains that a signal emanates from the centrosomes at anaphase onset and is transmitted to the cortex via the astral microtubules (Rappaport, 1961; White and Borisy, 1983; Harris and Gewalt, 1989; Devore *et al.*, 1989; Salmon and Wolniak, 1990; Rieder *et al.*, 1997). In the simplest form, this model contends that chromosomes are like the corpse at a funeral: they are the reason for the proceedings but play no active part (Mazia, 1961). This view recently gained support when cytokinesis was found to proceed unperturbed in meiotic grasshopper spermatocytes after removal of the chromosomes by micromanipulation (Zhang and Nicklas, 1996). Moreover, division occurs normally in chemically treated cells where only fragments of kinetochores are present in the mitotic spindle (Wise and Brinkley, 1997). However, while these results argue strongly against a role of chromosomal arms, it is difficult to rule out the contribution of kinetochore components that may have remained with microtubules in the micromanipulation experiment by Zhang and Nicklas (1996).

An alternative hypothesis proposes that the cleavage signal originates from the chromosomes (Margolis and Andreassen, 1993; Martineau *et al.*, 1995; Cao and Wang, 1996; Wheatley and Wang, 1996; Eckley *et al.*, 1997). This notion is supported largely by studies in



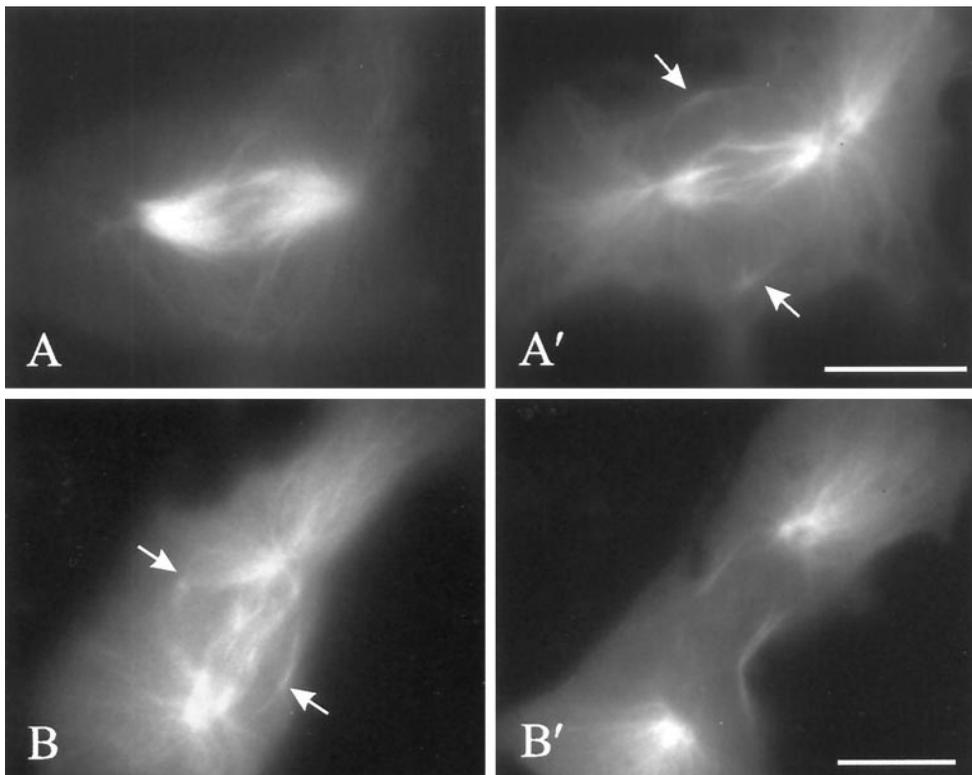
**Figure 7.** Correlation between microtubule bundles and furrowing. Cells were treated with ICRF-187 and microinjected with TAMRA-tubulin as in Figure 5. Panels A–C show cortical microtubule bundles in three separate cells. Microtubule bundles were always associated with the ingressing margin, including those misplaced (B, arrow) or formed ectopically (C, arrow). A similar situation was observed when cells were treated with nocodazole during anaphase. Panels D–G show a sequence taken from a cell 0, 10, 28, and 44 min after the addition of nocodazole. Often a small number of microtubule bundles persisted and remained associated with the furrow along a twisted path (arrows). Bars, 20  $\mu\text{m}$ .

cultured mammalian cells. For example, creation of a barrier between the cortex and the mitotic apparatus during late metaphase/early anaphase causes inhibition of cleavage (Cao and Wang, 1996). In addition, in cells with multipolar spindles, the pattern of cleavage correlates with the distribution of metaphase chromosomes and midzone microtubule bundles, rather than the position of spindle poles (Wheatley and Wang, 1996; Eckley *et al.*, 1997). Even in sand dollar embryos, there are indications that some signals may emanate from the nuclear region and complement astral stimulation (Rappaport, 1991b). Thus, it is possible that chromosome-mediated signaling represents a universal mechanism for spindle–cortex communications, while an additional strategy based on astral microtubules is used by large embryos to overcome the long distance between chromosomes and the cortex.

### *Spindle Interzone Plays a Critical Role in Cytokinesis*

In this study we prevented the separation of chromosomal arms and creation of the spindle interzone with a topo II inhibitor. This manipulation has no apparent effects on the integrity of spindle poles, the organization of astral microtubules, or other cellular processes such as the progression of mitosis, thus allowing us to address specifically the role of components associated with the spindle interzone in directing cell cleavage.

We discovered that, while cytokinesis initiated at the expected time, cleavage in most treated cells failed to localize along the equator as defined by the metaphase plate, which led to the formation of ectopic or grossly distorted furrows. In addition, while F-actin and myosin II are concentrated across the equator in control NRK cells (Fishkind and Wang, 1993), in topo



**Figure 8.** Development of microtubule puffs and bundles in the absence of cleavage. ICRF-187-treated cells were microinjected with fluorescent tubulin and treated with  $1 \mu\text{M}$  cytochalasin D at anaphase onset. Microtubule puffs formed subsequently as in control cells (A), followed by the development of cortical microtubule bundles 8 min after the addition of cytochalasin D (A', arrows), in the absence of cleavage activities. Panels B and B' show a separate cell treated similarly with ICRF-187 and cytochalasin D. Cortical microtubules were visible 8 min after the addition of cytochalasin D at anaphase onset (B, arrows). The cell started to cleave when cytochalasin D was subsequently removed from the medium (B'), at sites defined by the microtubule bundles. Bars,  $10 \mu\text{m}$ .

In inhibited cells these filaments become concentrated only near the lateral margins, suggesting that contractile signals and activities are focused in these regions. Our results indicate that, like *Echinoderm* embryos, the entire cortex of cultured cells is capable of undergoing cleavage upon reception of the appropriate signal (Rappaport, 1985). However, the meandering behavior of furrows in both ICRF-187- and nocodazole-treated cells suggests that proper progression of the furrow cannot be achieved by isolated sites of ingression but requires concerted contractions along the equator.

Given the apparently normal spindle poles and astral microtubules, it is difficult to explain the present results with the conventional model of cortical stimulation by astral microtubules. In addition, the abnormalities of cleavage cannot be explained entirely by the physical hindrance of cortical ingression by chromosomes (Mullins and Biesele, 1977), since distorted cleavage furrows can be found far away from chromosomes, and  $\sim 40\%$  of the furrows did cut successfully through the tangled chromosomes. Although cytokinesis may be affected by the inhibition of spindle

elongation, this inhibition should lead to a slightly shorter distance between spindle poles and the equatorial cortex and cannot easily account for the disruptive effects according to the conventional model. Together, our observations suggest that the effects of spindle interzone disruption dominate over any contribution that may be transmitted by the astral microtubules in specifying the site of cell cleavage.

#### *Interzonal Microtubules Stimulate Furrowing*

Given the physical separation between the spindle and the cortex, how does the spindle interzone specify the plane of cell division? The most intriguing finding in the present study is that, upon anaphase onset, a unique set of microtubules emanates from the region near tangled chromosomes toward the cortex. The site of contact between these microtubules and the cortex then becomes the site of cleavage. Thus, this set of puffing microtubules and associated motor molecules probably carry the signals for cytokinesis from the chromosomes to the cell cortex.

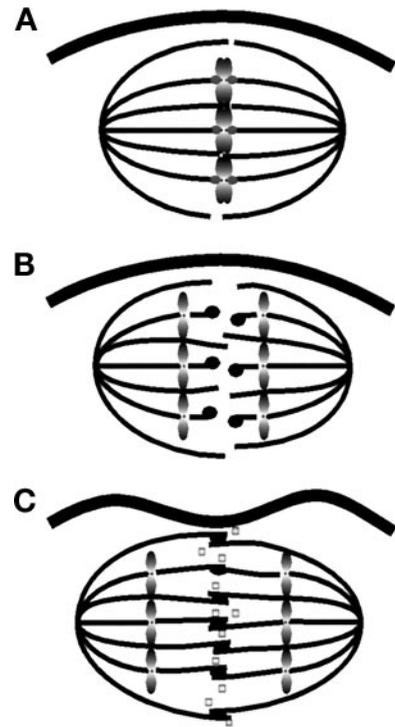
These puffing microtubules probably correspond to interzonal microtubules in control cells, which during early anaphase extend from separating chromosomes toward the equator (Mastronarde *et al.*, 1993). Microtubules from the two half-spindles then terminate and overlap near the equatorial cortex. In topo II-inhibited cells, the physical constraint imposed by tangled chromosomes likely forces these microtubules to puff laterally from the region occupied by the chromosomes, concentrating the cleavage activities to the lateral margins. In addition, the pattern of cleavage most likely reflects the angle at which these microtubules extend from the spindle. Mislocated or ectopic furrows arise when the microtubules extend along an acute angle and meet the cortex at a site away from the equatorial plane.

While these interzonal or interpolar microtubules have been examined in previous studies (Mastronarde *et al.*, 1993), little is known about their origin, composition, or dynamics. From our reconstructed images (Figure 6), they appear to be independent of microtubules associated with the spindle poles. Thus, these interzonal microtubules may arise *de novo* from the chromosomal region after anaphase onset, possibly as a result of chromosome-induced microtubule nucleation as observed *in vitro* (Heald *et al.*, 1996). However, it is also possible that part of these microtubules may derive from the extension or recruitment of pre-existing microtubules.

#### **Relationship between Midzone Microtubule Bundles and Furrowing Activities**

We noticed that after the initial contact between microtubule puffs and cortex, prominent microtubule bundles appeared at the cortex and remained associated with the cleavage furrow. These structures appear to incorporate both puff and polar microtubules (Figure 6). While in control cells similar microtubule bundles are found to span the equatorial region, in topo II-inhibited cells they are concentrated at the ingressing lateral margins, most likely as a result of the lateral extension of microtubule puffs that precede their formation.

The ability of midzone microtubule bundles to stimulate and maintain furrowing has been implicated in earlier observations (Rappaport and Rappaport, 1974; Kawamura, 1977), and supported by recent studies (Wheatley and Wang, 1996; Zhang and Nicklas, 1996). Since these bundles form even when cleavage is inhibited with cytochalasin D, they are not simply a result of the ingressing furrow, but more likely represent structures required for the maintenance of cleavage activities (see also Wheatley and Wang, 1996). The active role of cortical microtubule bundles is further supported by their constant association with meandering cleavage furrows in cells treated with nocodazole.



**Figure 9.** A hypothetical model for the signaling events of cytokinesis. During metaphase (A), inactive signaling molecules and associated microtubule bundling/motor molecules are concentrated near kinetochores or centromeres (gray dots). Upon anaphase onset (B), these molecules are activated and released from chromosomes. They migrate along interzonal microtubules that extend from separating chromosomes to the equatorial cortex (B, black dots), where they induce bundling of microtubules (C). The concentration of microtubule ends along the equator may help maintain a concentration of signals for cortical contraction (C, small squares).

Due to the extensive depolymerization of microtubules, it is likely that these bundles represent functional structures that resist the immediate action of nocodazole, rather than passive accumulation of free microtubules (of which there should be few in nocodazole-treated cells) created by the ingressing furrow.

Combining previous and present results, it is likely that at least part of the signals for cytokinesis are concentrated near the kinetochores at metaphase (Figure 9A), as a complex with microtubule motor proteins and factors that nucleate, bundle, and/or terminate microtubules. Several proteins, including TD60 (Andreassen *et al.*, 1991; Margolis and Andreassen, 1993), and INCENP (Cooke *et al.*, 1987; Earnshaw and Bernat, 1991; Eckley *et al.*, 1997; Mackay *et al.*, 1998), have been found to relocate from the kinetochore region to the equatorial cortex before the onset of cytokinesis. We propose that, after anaphase onset, these proteins dissociate from the kinetochores and migrate along a set of microtubules that extend from separating chromosomes to the equatorial cortex. Thereafter,

they interact with the cortex (Figure 9B) and induce the formation of microtubule bundles by cross-linking pre-existing microtubules or microtubules assembled de novo near the cortex (Figure 9C). The signal for cortical contraction may then be released from a concentration of microtubule ends.

In summary, in this study we have disrupted the organization of midzone microtubules by inhibiting chromosome separation. The resulting abnormalities in cleavage indicate that midzone microtubules play a dominant role in stimulating cytokinesis in NRK cells and that the normal configuration of spindle poles and polar microtubules is insufficient to ensure successful cleavage. In addition, we discovered that a unique set of microtubules, originating near separating chromosomes, may serve to mediate the transmission of cleavage signals and the formation of midzone microtubule bundles.

## ACKNOWLEDGMENTS

We would like to thank Dr. Anthony Imondi of Pharmacia (Oncology Division, Albuquerque, NM) for donating ICRF-187 and Drs. S. Doxsey (University of Massachusetts Medical Center, Worcester, MA), K. Fujiwara (National Cardiovascular Center Research Institute, Osaka, Japan), and D. Palmer (University of Washington, Seattle, WA) for supplying antibodies. We are also grateful to Dr. D. Fishkind (University of Notre Dame, South Bend, IN) for developing and sharing with us a fixation protocol for optimized myosin II preservation. Our research is supported by grant GM-32476 from the National Institutes of Health.

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