

Chromosomal Proteins and Cytokinesis: Patterns of Cleavage Furrow Formation and Inner Centromere Protein Positioning in Mitotic Heterokaryons and Mid-anaphase Cells

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Abstract. After the separation of sister chromatids in anaphase, it is essential that the cell position a cleavage furrow so that it partitions the chromatids into two daughter cells of roughly equal size. The mechanism by which cells position this cleavage furrow remains unknown, although the best current model is that furrows always assemble midway between asters. We used micromanipulation of human cultured cells to produce mitotic heterokaryons with two spindles fused in a V conformation. The majority (15/19) of these cells cleaved along a single plane that transected the two arms of the V at the position where the metaphase plate had been, a result at odds with current views of furrow positioning. However, four cells did form an additional ectopic furrow between the spindle poles at the open end of the V, consistent with the established view. To begin to address the mechanism of furrow assembly, we have begun a detailed study of the properties of the

chromosome passenger inner centromere protein (INCENP) in anaphase and telophase cells. We found that INCENP is a very early component of the cleavage furrow, accumulating at the equatorial cortex before any noticeable cortical shape change and before any local accumulation of myosin heavy chain. In mitotic heterokaryons, INCENP was detected in association with spindle midzone microtubules beneath sites of furrowing and was not detected when furrows were absent. A functional role for INCENP in cytokinesis was suggested in experiments where a nearly full-length INCENP was tethered to the centromere. Many cells expressing the chimeric INCENP failed to complete cytokinesis and entered the next cell cycle with daughter cells connected by a large intercellular bridge with a prominent midbody. Together, these results suggest that INCENP has a role in either the assembly or function of the cleavage furrow.

SUCCESSFUL cell division requires the orderly movement of sister chromatids to the spindle poles followed by the physical separation of the daughter cells. This latter event is termed cytokinesis. Recent years have seen dramatic advances in our understanding of how kinetochores interact with spindle microtubules to direct the chromosome movements in mitosis. Much less is understood about the positioning and assembly of the cleavage furrow that brings about cytokinesis (Rappaport, 1986).

At the beginning of cytokinesis, actin filaments and myosin become concentrated in a cortical band midway between the two spindle poles. Current models propose that

actin–myosin interactions induce localized cortical contraction, resulting in an invagination of the plasma membrane in the cleavage furrow (for reviews see Mabuchi, 1986; Salmon, 1989; Satterwhite and Pollard, 1992; Fishkind and Wang, 1995). The actomyosin system has an essential role in cytokinesis: its disruption by microinjection of antimyosin antibodies (Mabuchi and Okuno, 1977), myosin gene knockout (De Lozanne and Spudich, 1987), or treatment with actin-depolymerizing drugs (Aubin et al., 1981) results in incomplete or deficient cytokinesis.

Micromanipulation experiments on fertilized echinoderm eggs revealed that spindle asters have an essential role in stimulating cleavage furrow formation. The original evidence in support of this “astral stimulation” model was obtained by Rappaport (1961), who manipulated fertilized sand dollar eggs before the first division to form a torus by gently perforating the cell center (see diagram in Fig. 1A). Each toroidal cell proceeded to divide on one side, between the inner and outer cell surfaces, thereby producing a C-shaped, binucleate cell. At the next mitosis, this cell

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contained two spindles, and yet it formed three furrows, the third equidistant from the other two. It was proposed that the overlapping astral microtubules from the “back sides” of the two spindles somehow directed formation of the third furrow. Similar results were obtained when sea urchin eggs were manipulated so that they contained two mitotic spindles (see Fig. 1 A) (Salmon and Wolniak, 1990). In other experiments, removal of one or both asters early in mitosis blocked division (Hiramoto, 1956; Sluder et al., 1986), while microinjection of centrioles (Heidemann and Kirschner, 1975) caused localized furrowing in eggs. Furthermore, spermatocytes that contain a spindle without chromosomes can also assemble a functional cleavage furrow (Zhang and Nicklas, 1996). In addition to the asters, signals from the spindle midzone are also crucial for the stimulation of furrowing (Rappaport and Rappaport, 1983; Cao and Wang, 1996). This established view of cleavage furrow positioning has recently been challenged by studies in a mammalian cultured cell line in which it was found that heterokaryons with V- or Y-shaped spindles do not necessarily position cleavage furrows between all adjacent pairs of asters (Wheatley and Wang, 1996).

The biochemical mechanism by which the cleavage furrow becomes positioned at the cortical equator is presently unknown. Electron microscopy studies suggest that the accumulation of actin in a localized region of the cortex may help to position myosin motor molecules (Maupin and Pollard, 1986). However, it now seems likely that other proteins also have a role in contractile ring formation. For example, studies of both fertilized eggs and cultured somatic cells indicate that signals from the spindle midzone may be necessary for furrow placement (Rappaport and Rappaport, 1983; Cao and Wang, 1996).

The inner centromere protein (INCENP)¹ was discovered in a monoclonal antibody screen for novel chromosome scaffold proteins (Cooke et al., 1987). In mitosis, INCENP appears to move from the chromosome arms to become concentrated at centromeres during metaphase (Earnshaw and Cooke, 1991). During late metaphase or at the transition into anaphase, INCENP leaves the chromosomes, becoming associated with the spindle and the cell cortex at the presumptive cleavage furrow. These properties of INCENP led us to propose the existence of a class of proteins that we termed “chromosome passengers” (Earnshaw and Bernat, 1990). These proteins are associated with chromosomes during metaphase, but become associated with the spindle during anaphase (Cooke et al., 1987; Kingwell et al., 1987; Pankov et al., 1990; Andreassen et al., 1991; Yen et al., 1991; Rattner et al., 1992; Casiano et al., 1993; Rattner et al., 1993; Vernos et al., 1995; Wang and Adler, 1995). Two well-studied passenger proteins, INCENP and telophase disc protein of 60 kD (TD-60), both exhibit an early association with the presumptive cleavage furrow (Cooke et al., 1987; Andreassen et al., 1991; Earnshaw and Cooke, 1991; Margolis and Andreassen, 1993; Wheatley and Wang, 1996). One or both of these proteins might therefore be involved in furrow assembly, as proposed originally for INCENP (Cooke et al., 1987) and also for TD-60 (Martineau et al., 1995).

1. *Abbreviations used in this paper:* INCENP, inner centromere protein; Sf9, *Spodoptera frugiperda*; TD-60, telophase disc protein of 60 kD.

In the present study, we have examined two aspects of the mechanism of cytokinesis. First, we designed a test of the “astral stimulation” model of cleavage furrow positioning using a somatic cultured cell line. We used micromanipulation to generate mitotic heterokaryons in which two bipolar spindles had fused to form one V-shaped tripolar spindle. When these cells underwent cytokinesis, they typically did so by means of a single furrow that was positioned across the arms of the V. Thus, cultured mammalian cells do not invariably cleave midway between spindle asters. In a second series of experiments, we have examined the role of INCENP, a chromosome passenger protein, in cytokinesis. First, we have shown that INCENP concentrates in the presumptive cleavage furrow very early—even before cytoplasmic myosin II. Second, we have shown that the INCENP distribution in mitotic heterokaryons at anaphase mirrors the distribution of cleavage furrows. Finally, we have shown that when INCENP is tethered to the centromere regions of chromosomes throughout mitosis, a dominant disruption of cytokinesis is produced. Together, these experiments are consistent with the notion that chromosomal proteins such as INCENP may have a role in the assembly or function of the cleavage furrow in vertebrate somatic cells.

Materials and Methods

Buffers

KB buffer contains 10 mM Tris, pH 7.7, 0.15 M NaCl, 0.1% BSA. PBS contains 8.06 mM Na₂HPO₄, 1.47 mM KH₂PO₄, 137 mM NaCl, 2.7 mM KCl, 0.68 mM CaCl₂, 0.492 mM MgCl₂. The recipe for PHEM buffer used here contains 100 mM Pipes, pH 6.9, 10 mM Hepes, 10 mM EGTA, 2 mM MgCl₂.

Baculovirus Production of Recombinant INCENP

Recombinant baculoviruses expressing chicken INCENP were obtained by homologous recombination between a transfer vector construct (pVL1393; InVitrogen, San Diego, CA) that contained the INCENP open reading frame (these sequence data are available from Genbank/EMBL/DBJ under accession number Z25419) and linearized baculoviral genomic DNA (*Autographa californica* nuclear polyhedrosis virus expressing β -galactosidase across the site of cDNA integration: AcMNPV-lacZ). Recombination occurred in *Spodoptera frugiperda* (Sf9) host cells transfected with a mixture of plasmid and viral DNAs, according to a protocol developed in our laboratory. Recombinant viruses were isolated from the medium after 3–7 d and cloned by limiting dilution. Briefly, Sf9 cells were grown in 96-well plates until 50% confluent. Each plate was divided into four quadrants of 24 wells and all wells in each quadrant were infected with a single pool of diluted virus stock. Different quadrants received 10-fold serial dilutions of virus stock so that virus production could be assayed over a 1,000-fold range with only a single plate. After 1 wk, the culture medium was withdrawn from each well and kept as high titer virus stock and replaced with fresh medium containing Bluo-Gal (Boehringer Mannheim, Mannheim, Germany) to detect β -galactosidase activity. All wells were harvested by trituration of the medium with a pipette, and half of the resulting whole cell extract was dot blotted and probed with anti-INCENP monoclonal 3D3 (Cooke et al., 1987), which was detected by autoradiography after incubation of membranes with ¹²⁵I-labeled protein A. Clones positive for INCENP production and negative for β -galactosidase activity were assayed by SDS-PAGE (Laemmli, 1970) and immunoblotting (Earnshaw et al., 1984) to identify recombinants. Viruses were stored as high titer stocks (10⁸ pfu/ml) in the dark at 4°C.

Antibodies

Polyclonal Sera Recognizing INCENP. To isolate chicken INCENPs from baculovirus-infected Sf9 cells, the cells were swollen in hypotonic buffer

(7.5 mM Tris, pH 7.4, 40 mM KCl, 1 mM K-EDTA, 0.37 mM Spermidine, 0.15 mM Spermine) and lysed by Dounce homogenization in the presence of 0.1% digitonin. Nuclei were centrifuged in a microfuge and then resuspended in 1× SDS-PAGE buffer (Laemmli, 1970), sonicated, and boiled before loading on an SDS gel. Overproduced INCENP bands (~40 µg per gel) were cut out of the gel with a razor blade, frozen in a mortar filled with liquid nitrogen, and pulverized with a pestle. Immunization at 4-wk intervals and bleeds (10 d after each boost) were performed by Hazleton HRP, Inc. (Denver, PA).

Other Antibodies. Monoclonal antibody mAb 23, raised against chicken brain myosin, is specific for cytoplasmic myosin IIB (Conrad et al., 1991) and was a generous gift of Dr. Abigail Conrad (Kansas State University). Monoclonal antibody TU27B (isolated by S. Bender, a gift from Conly Reider, Wadsworth Institute, Albany, NY) was raised against polymerized, stabilized tubulin; antibody was obtained from tissue culture supernatant or as ascites fluid from mice injected with the hybridoma cells. Rat anti-mouse radixin (R21) was the gift of Prof. S. Tsukita (Kyoto University, Kyoto, Japan).

Although other investigators using different antimyosin antibodies have detected myosin associated with only 40–70% of cleavage furrows in HeLa cells (Maupin et al., 1994), mAb 23 allowed detection of myosin in 100% of the cleavage furrows of late anaphase and telophase chicken cells (Conrad et al., 1995).

Cell Culture

DU249 chicken hepatoma cells (Langlois et al., 1974) were grown in RPMI 1640 with 10% FBS at 37°C in 5% CO₂. Cells were subcultured every 3 d by trypsinization and replated at a 1:20 dilution. U2OS human osteosarcoma cells were grown in DME with 10% FBS at 37°C in 5% CO₂. Cells were subcultured every 3 d by trypsinization and replated at a 1:8 dilution.

Immunofluorescence

Mitotic DU249 cells were obtained by selective detachment (Tobey et al., 1967), which, unlike synchrony protocols involving drug treatment or cold incubation, does not disrupt spindle structure. After one wash in warm, serum-free RPMI 1640, cells were adsorbed to adhesion slides (Paul Marienfeld GmbH & Co., Bad Mergentheim, Germany) by incubation at room temperature for 7–10 min, followed by fixation for 2 min with 4% formaldehyde in PHEM. Populations obtained in this way were highly enriched for cells in metaphase and early anaphase. Antibody staining was performed as described previously (Earnshaw and Cooke, 1991). Briefly, fixed cells were washed 3× for 2 min in KB buffer containing 0.02% Triton X-100. Antibody was diluted in KB and incubated with cells for 30 min at 37°C. Three 2-min washes were performed at room temperature. Further incubations with secondary antibodies were done at 37°C. Biotinylated secondary antibodies (used at 1:500 dilution) were used to localize the anti-INCENP antibodies (biotinylated anti-rabbit; Vector Labs, Burlingame, CA). Streptavidin-Texas red (Life Technologies, Gaithersburg, MD) was used at 1:800 to detect the secondary antibodies. FITC-labeled anti-mouse antibodies (Vector Labs) were used at 1:100 to detect the antimyosin and at 1:50 to detect the anti-tubulin monoclonal antibodies. Slides were mounted in 25% glycerol in PBS. Diazido-bicyclo-octane (Sigma Chemical Co., St. Louis, MO) was added at 10 µg/ml to retard fading. Mounted slides were sealed with nail polish and scored immediately.

Mitotic U2OS cells were stained as described above, except that coverslips were fixed in 4% paraformaldehyde in PBS and post-fixed in methanol at –20°C to preserve microtubule structure.

Micromanipulation to Produce Mitotic Heterokaryons

Cells cultured on etched locator coverslips (Bellco Glass, Vineland, NJ) were allowed to grow to 75% confluency. Cultures were examined using an inverted microscope (model Diaphot; Nikon Inc., Melville, NY) equipped with a 60× NA 1.3 objective (model Ph3; Nikon Inc.). Cells were maintained at 37°C using a microscope stage incubator (model NP-2; Nikon Inc.) before micromanipulation and throughout observation. Closely apposed cells in prometaphase were identified, and their locations were noted. Later, each member of several adjacent pairs of metaphase cells was injected with PBS near the point of contact with its neighbor using a needle drawn from a 1.5-mm bore capillary. (Needles drawn from smaller bore capillaries were ineffective at inducing fusion.) A micromanipulator (model MM188; Narashige USA Inc., Greenvale, NY) was used to position the needle, and a microinjector (model PLI 188; Nikon, Inc.)

was used to deliver a pulse of PBS. Most cells treated in this way were unaffected by the treatment and traversed mitosis normally. Occasionally, cells treated in this way fused to form mitotic heterokaryons. Those heterokaryons were followed by phase contrast microscopy through the subsequent anaphase and telophase. Phase contrast images were collected using an MPI CCD2 camera (Dage MTI, Michigan City, IN) and converted into TIFF files using a Perceptics PixelPipeline card under the control of a customized Adobe Photoshop plug-in module (Mountain View, CA). In some cases, cells that were just beginning to undergo cytokinesis were fixed and processed for indirect immunofluorescence, as described above.

Expression of a Form of INCENP That Is Tethered at Human Centromeres

The CENP-B:INCENP chimera was constructed using PCR to generate a unique SacII restriction site at INCENP position 174 bp. The first 474 nucleotides of CENP-B were then cloned upstream in frame at this newly created site. The resultant construct CENP-B_{1–158}:INCENP_{43–839}, expressed in pECE (Ellis et al., 1986), contained the CENP-B centromere targeting domain (Pluta et al., 1992) and an INCENP deletion construct recently shown to be unable to go to centromeres. The DNA was purified on CsCl density gradients (Ausubel et al., 1991) and transfected into JW HeLa cells as previously described (Mackay et al., 1993) onto duplicate coverslips. Cells were fixed 24 or 48 h after transfection for 5 min at –20°C in MeOH. Immunofluorescence was performed as described above except using a chicken-specific anti-INCENP antibody (RabC) (Mackay et al., 1993). Both full-length INCENP and CENP-B_{1–158}:GFP transfectants were used as controls.

Results

Cleavage Furrows Do Not Always Form Midway Between Asters in Cultured Human Cells

We have performed a test of the classic “astral stimulation” hypothesis that furrows always form midway between asters (Rappaport, 1986) in a somatic cultured cell line. We found that needle perforation of adjacent mitotic human U2OS osteosarcoma cells would occasionally result in cell fusion to form mitotic heterokaryons. Fig. 1 provides a schematic representation of our experiment together with a diagram of two important studies of cytokinesis in echinoderm eggs (Rappaport, 1961; Salmon and Wolniak, 1990). In these studies, it was found that furrows form midway between asters, regardless of whether or not the asters are connected by a mitotic spindle (Fig. 1 A, *open arrows*). Our results obtained in somatic heterokaryons were different.

Mitotic heterokaryons frequently adopted a V-shaped spindle conformation with two closely apposed poles and two separated poles (Fig. 1 B). The two adjoined spindles organized their attached chromosomes into a single bent metaphase plate (Fig. 2, *0 min*). These V-shaped spindles appeared to be fully functional: at anaphase onset, the sister chromatids separated along the two arms of the V (Fig. 2, subsequent time points).

In 15 of 19 heterokaryons followed, cytokinesis involved a single furrow that formed across the plane of the original metaphase plate. More rarely (4/19 heterokaryons followed), an ectopic furrow appeared between the two spindle poles at the open end of the V (Fig. 2, *right, white arrow*). Closer examination of the phase contrast micrographs revealed that the behavior of heterokaryons during cytokinesis appeared to correlate with the appearance of the cytoplasm between the two spindle poles at the open end of the V. Typically, this had a granular appearance resembling the cytoplasm outside the spindle (Fig. 2, *left*). In the

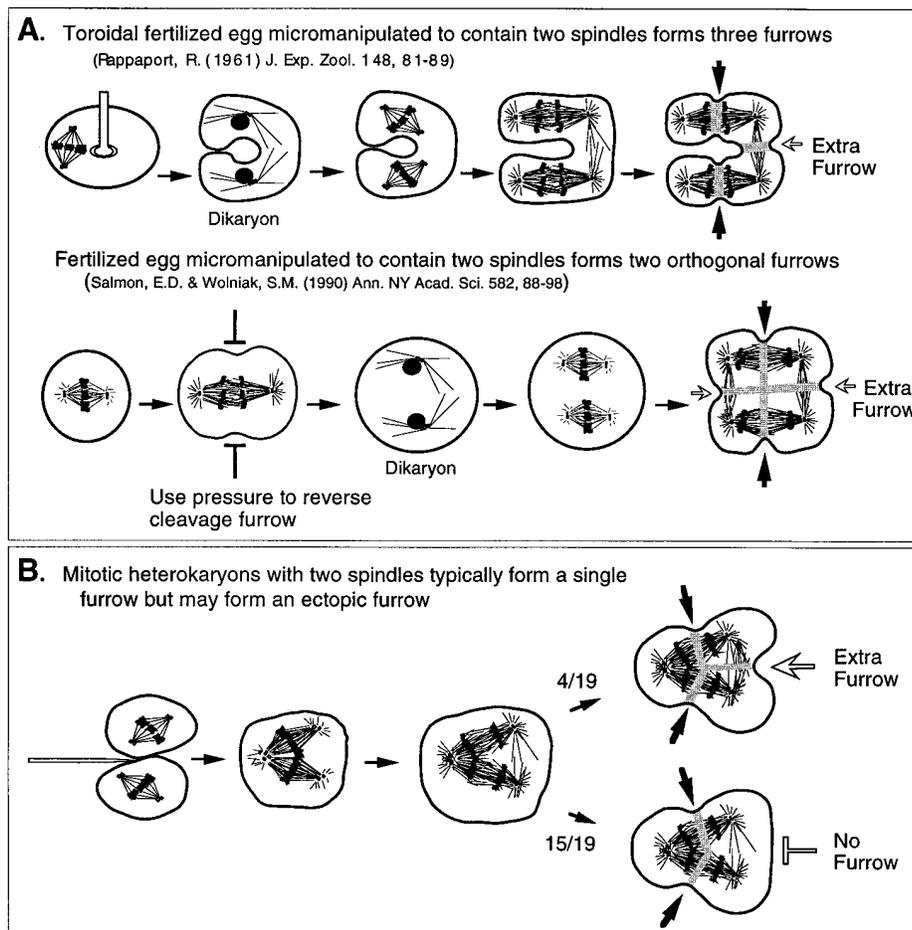


Figure 1. Relationship between astral position and furrow assembly in embryonic and somatic cells. (A) *Top:* A sand dollar zygote was manipulated into a toroidal shape by pressing with a blunt needle (Pappaport, 1961). Cytokinesis produced a tubular binucleate cell. During the following mitosis, this single cell contained two mitotic spindles arranged in tandem. During the subsequent cytokinesis, furrows formed above each spindle midzone. An additional ectopic furrow formed between the two adjacent asters that were oriented “back-to-back” and not connected by a spindle midzone (*hollow arrow*). *Bottom:* Cytokinesis was reversed in a fertilized sea urchin egg by the application of hydrostatic pressure (Salmon and Wolniak, 1990). The egg subsequently traversed interphase as a dikaryon, which formed two independent spindles during the next mitosis. After completion of anaphase, two orthogonal cleavage furrows formed. In this case, an ectopic furrow formed between two pairs of adjacent asters that were oriented “side-by-side” and not connected by a spindle midzone (*hollow arrows*). The outcome in both cases was cleavage giving rise to four diploid progeny cells. (B) Two adjacent mitotic U2OS cells were punctured with a microneedle

at the point of contact. These cells fused to form a heterokaryon containing two mitotic spindles that fused to make a V-shaped unit with a single bent metaphase plate. These cells initiated anaphase normally with the chromatids segregating into three masses. At cytokinesis, one furrow always formed across the middle of the V above the position where the metaphase plate had been (*solid arrows*). In most cells only this furrow formed; however, in four cells a second furrow formed between the adjacent asters at the open end of the V. Thus, although furrows can form between asters that are oriented “side-by-side” and not connected by a spindle midzone, this is not the most common outcome in these cells.

rare cells that did form ectopic furrows, the cytoplasm between two spindle poles at the open end of the V adopted a phase-lucent appearance resembling that between the separating sister chromatids on the spindle axes (Fig. 2, *right*). This appearance may reflect the development of an organized spindle midzone that excludes cytoplasmic organelles from the region. It has recently been argued that the spindle midzone is a crucial determinant of cleavage furrow assembly and function (Cao and Wang, 1996; Wheatley and Wang, 1996).

Two conclusions may be drawn from this experiment. First, in human U2OS cells, cleavage furrows can form between adjacent asters that are not connected by a fully formed spindle with associated chromosomes. Second, cleavage furrows typically do not form between adjacent asters in this cell type unless they are connected by a spindle bearing chromosomes. Thus, our study and a recently published study of rodent cells (Wheatley and Wang, 1996) reveal both similarities and differences in the regulation of cleavage furrow formation between fertilized eggs and somatic cultured cell lines.

Human INCENP Leaves the Chromosomes at Anaphase

Little is known about the role in cytokinesis of proteins other than the actomyosin complex. Because mitotic heterokaryons provide a novel system for the study of cytokinesis, we decided to use this system to probe the role of the INCENP chromosome passenger protein (Cooke et al., 1987; Earnshaw and Bernat, 1990) in the process. Polyclonal antibodies raised against chicken INCENPs expressed in Sf9 cells recognized both chicken and human INCENPs, as shown by immunoblotting of purified chromosomes and cell extracts (Fig. 3). The two INCENP polypeptides detected in chicken mitotic chromosomes (Fig. 3, lane *c*) apparently result from alternative splicing (Mackay et al., 1993). In contrast, only a single INCENP band is seen in immunoblots of human mitotic chromosomes (Fig. 3, lane *h*). This single polypeptide is also seen in total cell lysates (Fig. 3, lane *T*) and in a crude nuclear fraction (Fig. 3, lane *N*), but not in a crude cytosolic fraction (Fig. 3, lane *Cy*).

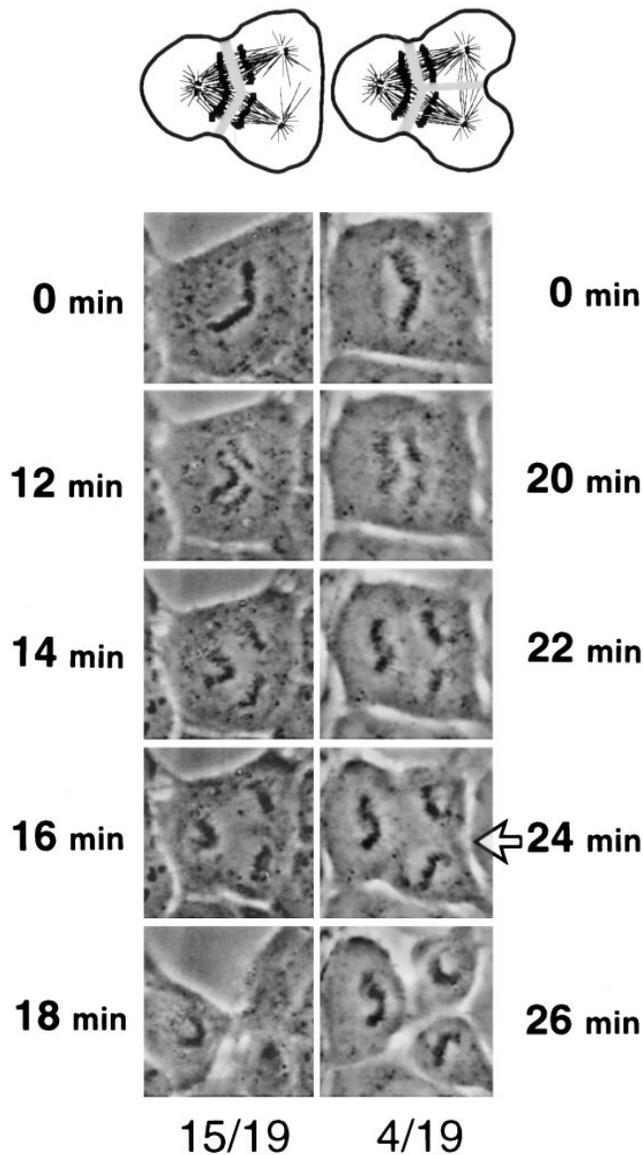


Figure 2. Adjacent asters usually do not direct cleavage furrow formation in somatic heterokaryons. Neighboring metaphase U2OS cells were microinjected at their point of contact and allowed to fuse, as depicted in Fig. 1. The heterokaryons were then observed as they progressed through the subsequent mitosis. (0 min) Chromosomes congress to a bent metaphase plate. (12, 20 min) Anaphase initiates with the metaphase plate resolving into three separate masses of chromatids. (16, 24 min) Separation of the chromatids is completed. A phase-lucent zone is visible along the axis of chromosome separation for both heterokaryons. An additional phase-lucent zone developed in 4 of 19 heterokaryons (right; 22, 24 min, white arrow). (18 min; left) In 15 of 19 cells followed, cytokinesis involved a single furrow over the spindle midzones at the location of the bent metaphase plate. (24, 26 min; right) In four cells, cytokinesis involved a furrow at the location of the bent metaphase plate plus an ectopic furrow located between the two distal spindle poles (white arrow).

Immunofluorescence localization studies with this antiserum confirmed that in interphase human U2OS cells, the distribution of INCENP across the cell cycle resembled that described previously for chicken cells (Earnshaw and Cooke, 1991). Notably, INCENP was associated with the

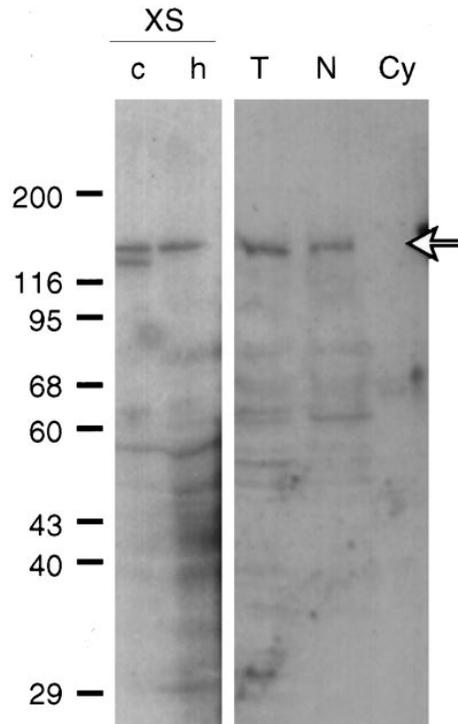


Figure 3. A single human INCENP is detected in purified chromosomes and cell nuclei. (Lanes *c* and *h*) Purified chromosomes from DU249 cells (chicken; lane *c*) or U2OS cells (human; lane *h*) were resolved on a 7.5% polyacrylamide gel before transfer to nitrocellulose. (Lanes *T*, *N*, and *Cy*) Human U2OS cells were lysed in SDS sample buffer and total protein was resolved on a 7.5% polyacrylamide gel (lane *T*) followed by blotting to nitrocellulose. Cells were fractionated by Dounce homogenization into nuclei (lane *N*) and cytoplasm (lane *Cy*) before gel electrophoresis and transfer to nitrocellulose. Blots were probed with cross-reactive polyclonal antiserum raised against recombinant chicken INCENP and detected using ¹²⁵I-labeled protein A. The positions of molecular weight standards on Ponceau S-stained membranes are indicated, as is the position of the major human immunoreactive band (arrow).

spindle midzone and the cell cortex at the site of future cleavage furrow formation (the presumptive cleavage furrow) from the onset of anaphase through the completion of cytokinesis (data not shown). Thus, both human and chicken INCENPs undergo similar movements in mitosis.

INCENP Distribution Correlates with Furrowing in Mitotic Heterokaryons

As described above, a few mitotic heterokaryons formed an ectopic furrow at the open end of the V-shaped spindle, consistent with the results of studies of echinoderm eggs. We wished to investigate whether this region had formed a spindle midzone complete with INCENP staining despite the prior lack of chromosomes between these poles. We therefore examined the status of the microtubules and INCENP at the open end of the V by indirect immunofluorescence. Cells were fused by needle injection as described above and then monitored for their subsequent progress through mitosis. Once furrowing had initiated, they were fixed and processed for immunodetection of both INCENP and tubulin. Two contrasting examples

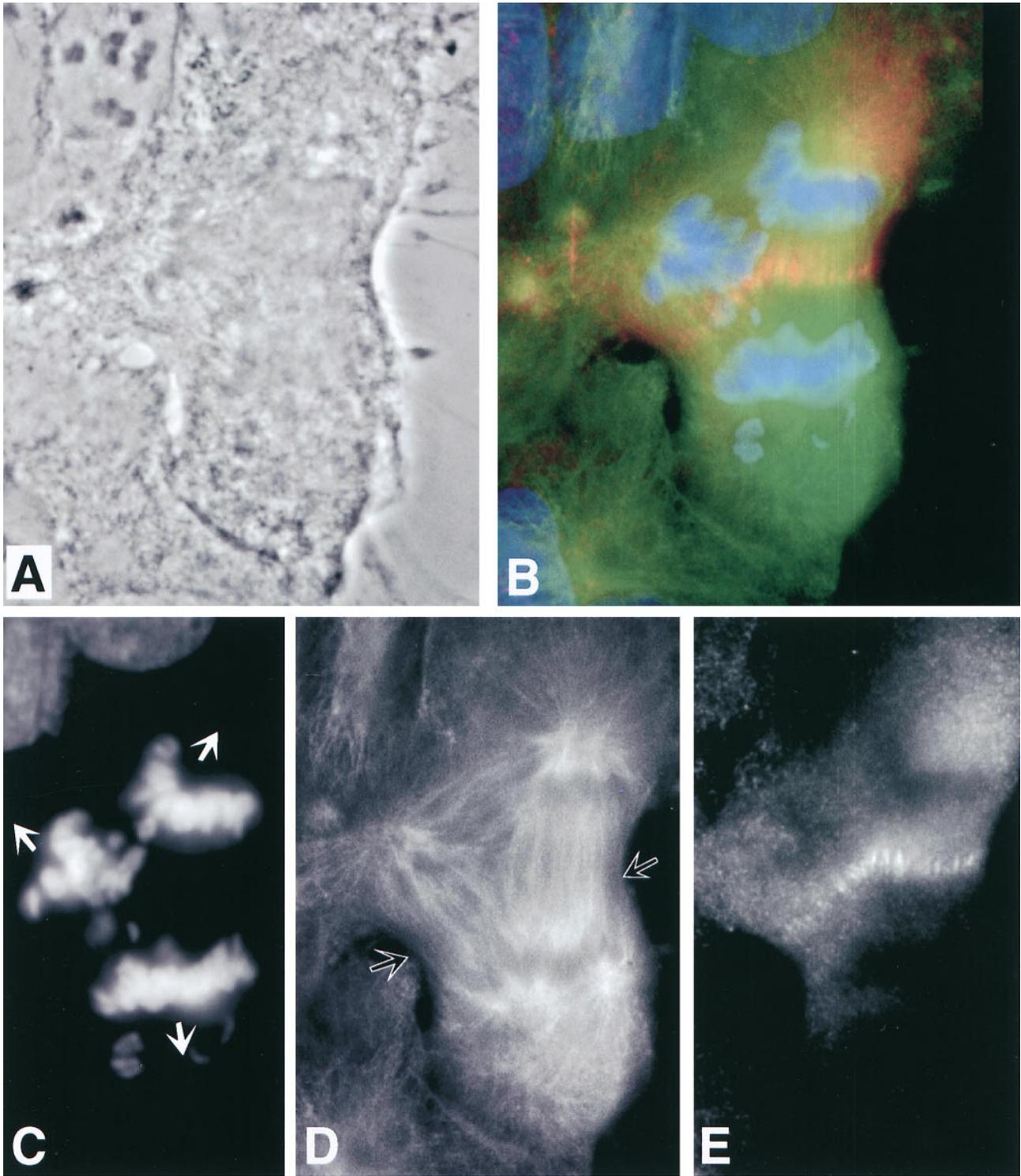


Figure 4. INCENP distribution correlates with sites of furrowing in a mitotic heterokaryon undergoing a triradial mitosis without an ectopic furrow. Human U2OS cells were fused by needle puncture, allowed to progress through mitosis until the commencement of furrowing, and then fixed and stained to localize INCENP. (A) Phase contrast image of the fixed cell. (B) Simultaneous localization of INCENP (red), tubulin (green), and the chromosomes (blue). (C) The separated sister chromatids. Arrows indicate the direction of chromatid movement. (D) The V-shaped spindle. Arrows indicate the axis of cleavage. (E) Localization of INCENP, with staining limited to the region above the original spindle midzone.

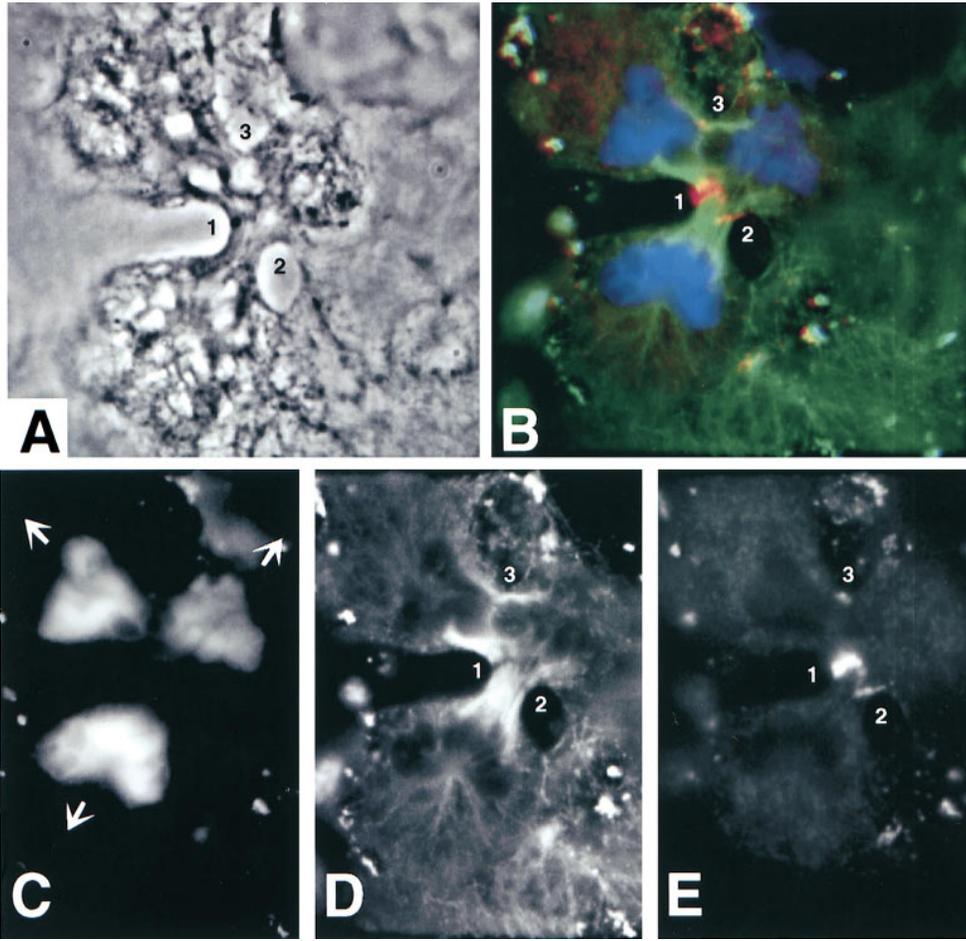


Figure 5. INCENP distribution correlates with sites of furrowing in mitotic heterokaryon undergoing a triradial mitosis with an ectopic furrow. (A) Phase contrast image of the fixed cell. This cell shows three sites of furrowing. These are numbered in order of the degree of microtubule bundling (1 = strongest, 3 = weakest). (B) Simultaneous localization of INCENP (red), tubulin (green), and the chromosomes (blue). (C) The separated sister chromatids. Arrows indicate the direction of chromatid movement. (D) The distribution of microtubules. (E) Localization of INCENP. All furrows have associated INCENP staining.

from this study are shown in Figs. 4 and 5. Although the cells were harvested before completion of cytokinesis out of necessity, video microscopy of the living cells revealed that the cell in Fig. 4 was dividing along a single plane, while that in Fig. 5 was in the process of forming an ectopic furrow.

The cell in Fig. 4 is oriented with the open end of the V-shaped spindle uppermost. The arrows in *C* indicate the direction of chromatid movement. The arrows in *D* indicate the location of the cleavage furrow (observed under phase contrast microscopy). At the time of fixation and staining, this cell was dividing along a single cleavage plane, thereby placing it in the majority class in Fig. 2. The cell shows a single band of INCENP staining located at the site of the original bent metaphase plate. There is no clear evidence of furrowing at the open end of the V-shaped spindle, despite the presence of antiparallel microtubules bridging the two spindle poles (Fig. 4, *D*).

At the time of fixation and staining, the cell of Fig. 5 was undergoing a multiple cleavage with an ectopic furrow, thereby placing it in the minority class in Fig. 2. Cleavage in this cell is complex, with three intercellular bridges forming (numbered 1–3 in the figure). This cell also shows a complex pattern of INCENP staining, with most INCENP concentrated in furrow 1 and less in furrow 2. A small focus of INCENP staining colocalizes with the minor midbody forming at position 3. Thus, in the two heterokaryons shown in

Figs. 4 and 5, there is a strong correlation between the INCENP distribution and the position of the cleavage furrow.

INCENP Staining in Naturally Occurring Cells with Abnormal Spindle Morphology

With the microinjection protocol for cell fusion, only a limited number of cells can be characterized with respect to their INCENP staining patterns. To obtain further examples of cells undergoing multipolar mitosis, we also examined the distribution of INCENP in untreated cells undergoing aberrant multipolar mitoses. We scanned cultures of human U2OS cells for those rare cells undergoing tripolar cytokinesis. As predicted from the results of the micromanipulation study, INCENP was associated with all cleavage furrows in every instance of spontaneous tripolar cleavage observed, both early in cytokinesis and nearer the completion of cell division (49 of 2,000 mitotic cells scored). Perhaps more informative were the few cases where the chromosomes had separated into three masses, but only a single cleavage furrow had formed. In these cells, INCENP was found between chromatin masses only when a spindle midzone (Fig. 6, *A–C*) or midbody (Fig. 6, *D–F*) was present. Overlapping microtubules between chromatin masses that did not direct furrow formation were not decorated with INCENP and appeared to be relatively disorganized (Fig. 6 *E*). Thus, in naturally occur-

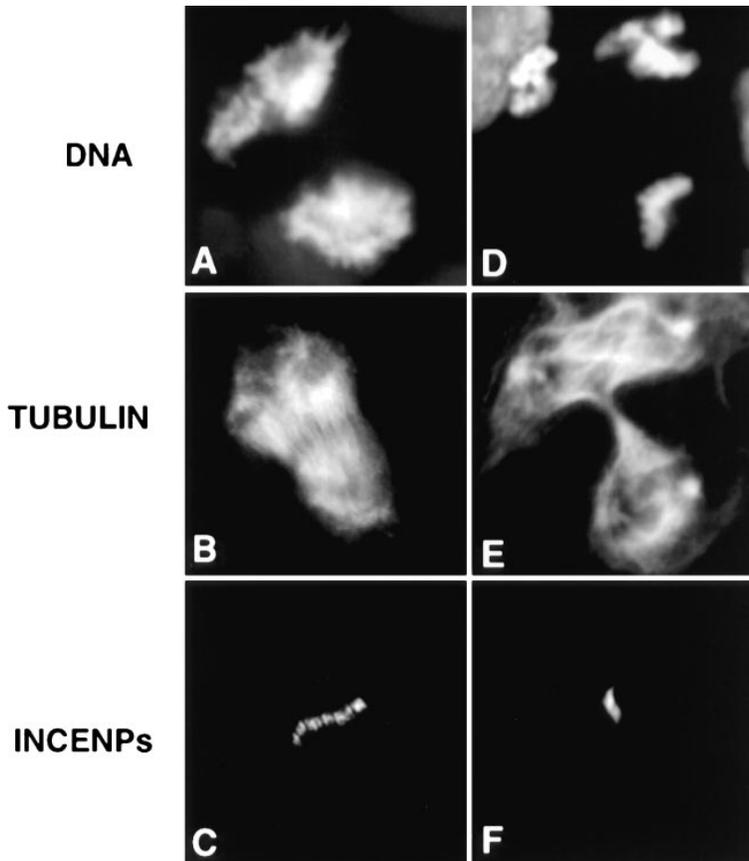
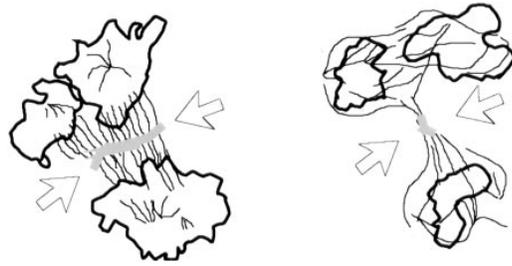


Figure 6. INCENP accumulates on organized spindle midzone microtubules but not on overlapping astral microtubules. Untreated U2OS cells with three masses of chromatids were fixed and stained for DNA, tubulin, and INCENP. (A) An anaphase cell with three masses of chromatids. (B) Microtubules formed a large and well-organized spindle midzone. (C) INCENP concentrated at the spindle midzone. (D) A telophase cell with three reforming nuclei. (E) Spindle midzone microtubules are collapsed under the advancing cleavage furrow into a single midbody structure. In the upper portion of the cell, microtubules are overlapping and unorganized, and no additional cleavage furrow is seen. (F) INCENP concentrated at the midbody but not on the disorganized microtubules at the top.

ring multipolar mitoses, as in mitotic heterokaryons, the position of INCENP in dividing cells correlates strongly with sites of furrowing.

INCENP Moves to the Spindle Midzone and the Cleavage Furrow Early in Anaphase

We showed previously that INCENP localizes to the inner surface of the cell membrane at the presumptive cleavage furrow before the onset of furrowing (Earnshaw and Cooke, 1991). This suggested that INCENP might be involved in very early events of furrow formation, perhaps even before localization of the contractile apparatus. This notion was consistent with the correlation between INCENP distribution and the sites of cleavage in heterokaryons. To further explore this possibility, we have investigated the relative timing of arrival of INCENP and two well-characterized components of the contractile apparatus at the presumptive cleavage furrow. We first stained populations of anaphase cells for both INCENP and myosin heavy chain (Fig. 7). Cells were also stained with 4',6-diamidino-2-phe-

nylindole so that the distance between the separating sister chromatids could be used to estimate the time elapsed since the onset of anaphase. Chicken DU249 cells (Langlois et al., 1974) were chosen for this study because they have a short cell cycle (doubling time, 18 h) and become extensively rounded up in mitosis. The presumptive cleavage furrow is visualized much more readily in round cells than it is in flat cells. This allowed the observation of the earliest cortical events of cytokinesis, e.g., the recruitment of cortex-associated proteins. By assessing the degree of separation of the chromatin masses, the INCENP and myosin staining patterns, and the presence or absence of a furrow, we were able to divide the 717 anaphase cells scored into four distinct stages (Table I).

In earliest anaphase (Fig. 7, *Stage I*), INCENP had moved to the spindle midzone, where it decorated the overlapping spindle midzone microtubules. No INCENP staining was detected at the cell cortex. At this time, myosin staining was diffusely cytoplasmic, and no furrow could be detected. Cells at the next stage of anaphase (Fig. 7, *Stage II*) showed a clear and unambiguous cortical ring of INCENP,

Table I. INCENP Concentrates in the Presumptive Cleavage Furrow before Myosin Heavy Chain in Anaphase Cells

Anaphase stage	INCENP staining	Myosin staining	Chromatid separation μm	Furrowing	Number
I Earliest anaphase	Spindle	Cytoplasmic	<2	No	47/717 (6.5%)
II Early anaphase	Spindle + Equatorial cortex	Cytoplasmic	2–4	No	102/717 (14%)
III Mid-anaphase	Spindle + Equatorial cortex	Cytoplasmic + Equatorial cortex	3–5	No	215/717 (30%)
IV Late anaphase	Spindle + Furrow	Cytoplasmic + Furrow	>4	Yes	353/717 (49%)

which coincided with the presumptive cleavage furrow. Again, no furrowing was detected in these cells. Myosin remained diffuse and cytoplasmic at this stage, with some staining of cortical patches. As the cells passed into mid-anaphase (Fig. 7, *Stage III*), INCENP remained concentrated at the cell equator, and myosin staining was unmistakable at the presumptive cleavage furrow. In most cells, myosin accumulation was accompanied by a cell shape change from round to oval, which was likely the conse-

quence of cortical actomyosin contractile activity. However, we have also observed myosin concentration in the presumptive furrow region of cells that showed no signs of furrowing. All stage III cells had an obvious accumulation of INCENP in the presumptive cleavage furrow. When cleavage began in late anaphase (Fig. 7, *Stage IV*), myosin staining coincided with INCENP staining at the furrow. These results are summarized in Table I.

We might have expected to observe a population of anaphase cells in which myosin was concentrated in the presumptive cleavage furrow before localization of INCENP. Of the 717 anaphases scored, none showed this behavior. Our results demonstrate that INCENP precedes the bulk of the cytoplasmic myosin to the cell equator and suggest that it may be one of the first components recruited to the site of cleavage furrow formation.

Radixin is an actin-capping protein that binds to the barbed end of actin filaments (Tsukita et al., 1989) and is found at the cell surface, where it has been reported to concentrate at the cleavage furrow during telophase (Sato et al., 1991). To further characterize the timing of INCENP recruitment to the cell cortex, we compared the localization of radixin and INCENP in 206 stage I–stage IV anaphase cells. We observed patches of cortical staining with the antiradixin antibody in all stages of anaphase, but concentration of radixin at the cleavage furrow was only seen in the 85 stage IV cells scored (Fig. 8). Thus, although radixin is detected at the cell surface throughout anaphase, the protein appears to concentrate at the cleavage furrow after INCENP and myosin heavy chain, a pattern that may reflect the timing of deposition of actin filaments at the cleavage furrow.

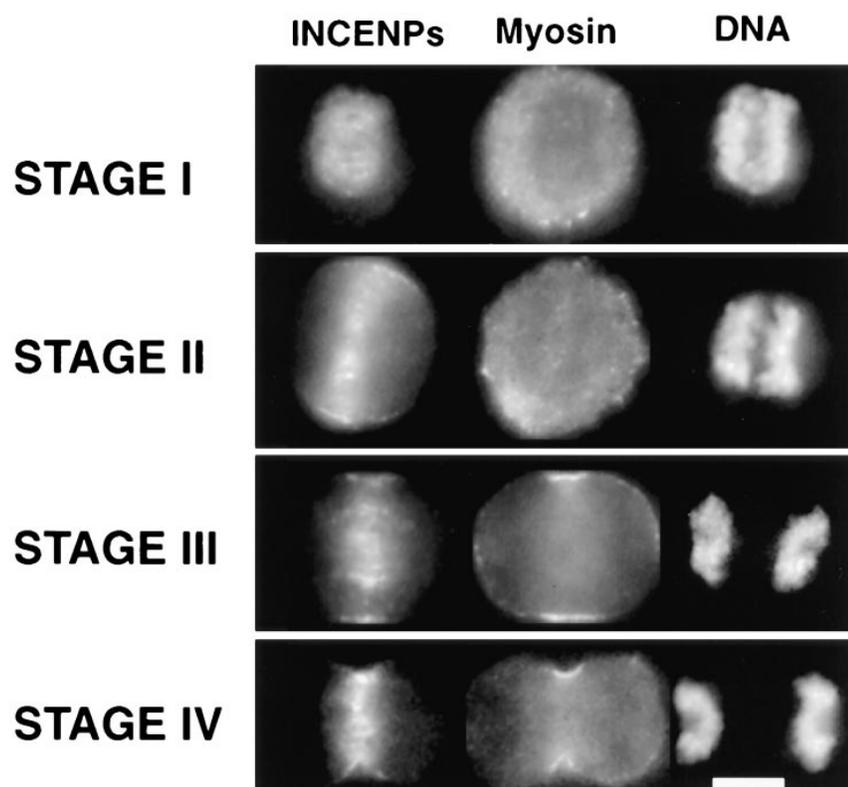


Figure 7. INCENP localizes to the cleavage furrow before cytoplasmic myosin in early anaphase cells. Mitotic cells were fixed and stained for INCENP, myosin, and DNA. Separate stages of anaphase were ordered by cell shape, the distance between the separating sister chromatids, and the relative distribution of INCENP and myosin. The stages are defined as follows: *STAGE I*, round cell with <2- μm separation between sister chromatids and with neither INCENP nor myosin at the equatorial cortex; *STAGE II*, round cell with 2–4- μm separation between sister chromatids. INCENP is concentrated at the equatorial cortex while myosin is still diffuse or in patches; *STAGE III*, oval cell with 3–5- μm separation between sister chromatids. Both INCENP and myosin are concentrated in the equatorial cortex; *STAGE IV*, elongated, furrowing cell with >4- μm separation between sister chromatids. Both INCENP and myosin are concentrated in the advancing furrow. Bar, 5 μm .

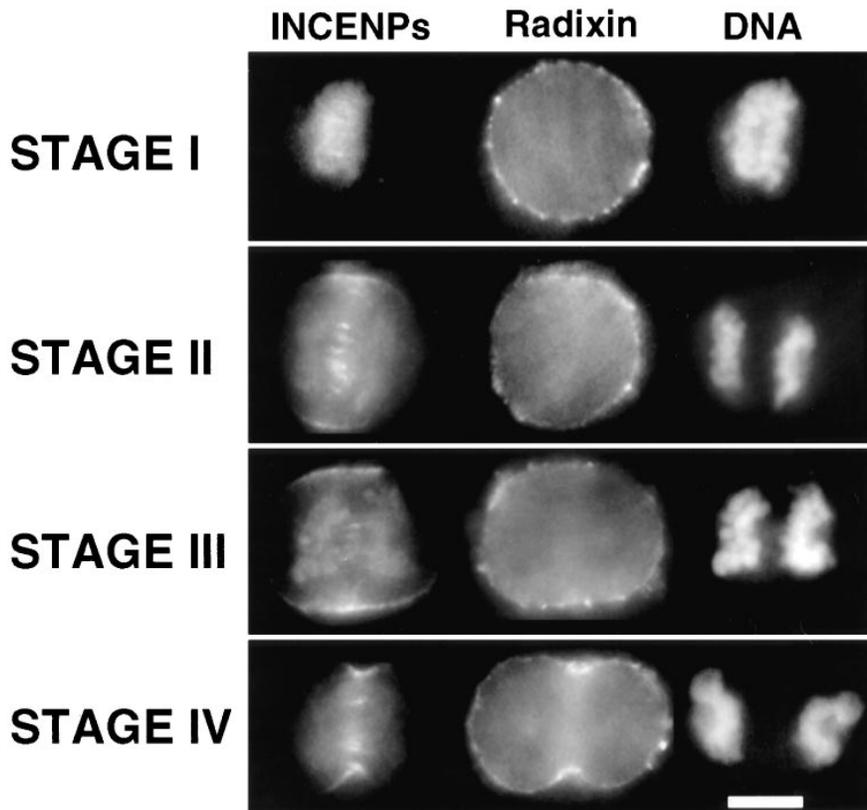


Figure 8. Radixin concentrates at the equatorial cortex only when noticeable furrowing has occurred. Mitotic cells were fixed and stained for INCENP, radixin, and DNA. Separate stages of anaphase were ordered by cell shape and the distance between separating sister chromatids, as described in the legend to Fig. 7. INCENP first concentrates at the equatorial cortex in stage II cells. In contrast, radixin is found in patches throughout the cortex of the cell in stages I–III and does not concentrate at the equatorial cortex until furrowing is well established (STAGE IV). Bar, 5 μ m.

Artificial Targeting of a Mutant INCENP to Centromeres Disrupts the Completion of Cytokinesis

Direct evidence for the functional involvement of INCENP in cytokinesis was obtained during the course of a study in which we were examining the transfer of INCENP from the chromosomes to the mitotic spindle during anaphase. We had previously shown that deletion of the amino-terminal 42 amino acids from chicken INCENP abolished the ability of the protein (termed INCENP_{43–839}) to transfer to the spindle at the metaphase–anaphase transition (Mackay et al., 1993). We subsequently found that this mutant INCENP also failed to concentrate at centromeres during metaphase (Mackay, A.M., and W.C. Earnshaw, unpublished observation).

To examine the relationship between centromere targeting and spindle transfer, we have forced INCENP_{43–839} to target to centromeres by fusing it to the centromere-targeting domain (residues 1–158) of CENP-B (Pluta et al., 1992). We had previously mapped the distribution of both CENP-B and INCENP in mitotic chromosomes by immunoelectron microscopy (Earnshaw and Cooke, 1991) and found that both proteins are broadly distributed throughout the heterochromatin beneath the kinetochore. Thus, CENP-B_{1–158} would be expected to target INCENP_{43–839} to roughly the correct region of the centromere. This mutant INCENP functioned as predicted with respect to centromere targeting, but it turned out to have a very unexpected phenotype—dominant disruption of the completion of cytokinesis.

Fig. 9 A shows that CENP-B_{1–158}:INCENP_{43–839} does target to centromeres where it remains tethered throughout

all stages of the mitotic cycle (Fig. 9, B and C). At first glance, the expression of CENP-B_{1–158}:INCENP_{43–839} appeared to have no deleterious effect in HeLa cells, since at 24 h after transfection, cells were seen in all stages of normal mitosis. However, when cultures were examined at 48 h after the transfection, a striking result was obtained. Initially, when screening by immunofluorescence microscopy, we noticed numerous pairs of mitotic cells (Fig. 9 D). Observation of these cells under phase contrast revealed a unique phenotype: the paired cells were joined by substantial intracellular bridges in which prominent midbody material could be seen (Fig. 9, D, E, and F). This midbody material did not stain with antibodies to either tubulin or INCENPs. Thus, tethering INCENP_{43–839} to the centromere during mitosis produces a dominant disruption of cytokinesis at a stage midway through the process (i.e., with an established midbody, but before the constriction of that midbody into a compact intercellular bridge).

In control experiments, expression of either CENP-B_{1–158} (Pluta et al., 1992) or INCENP_{43–839} (Mackay, A.M., and W.C. Earnshaw, unpublished observation) independently had no deleterious effects on mitotic progression. Expression of CENP-B_{1–158}:INCENP_{43–839} did not disrupt the normal localization of CENP-B to centromeres (data not shown). Furthermore, INCENP half-molecules, which concentrate at centromeres without the aid of the CENP-B targeting sequence, also disrupt cytokinesis (Mackay, A.M., and W.C. Earnshaw, unpublished observation). This experiment thus provides compelling evidence that expression of a mutant INCENP in cultured cells can result in a specific disruption of the completion of cytokinesis.

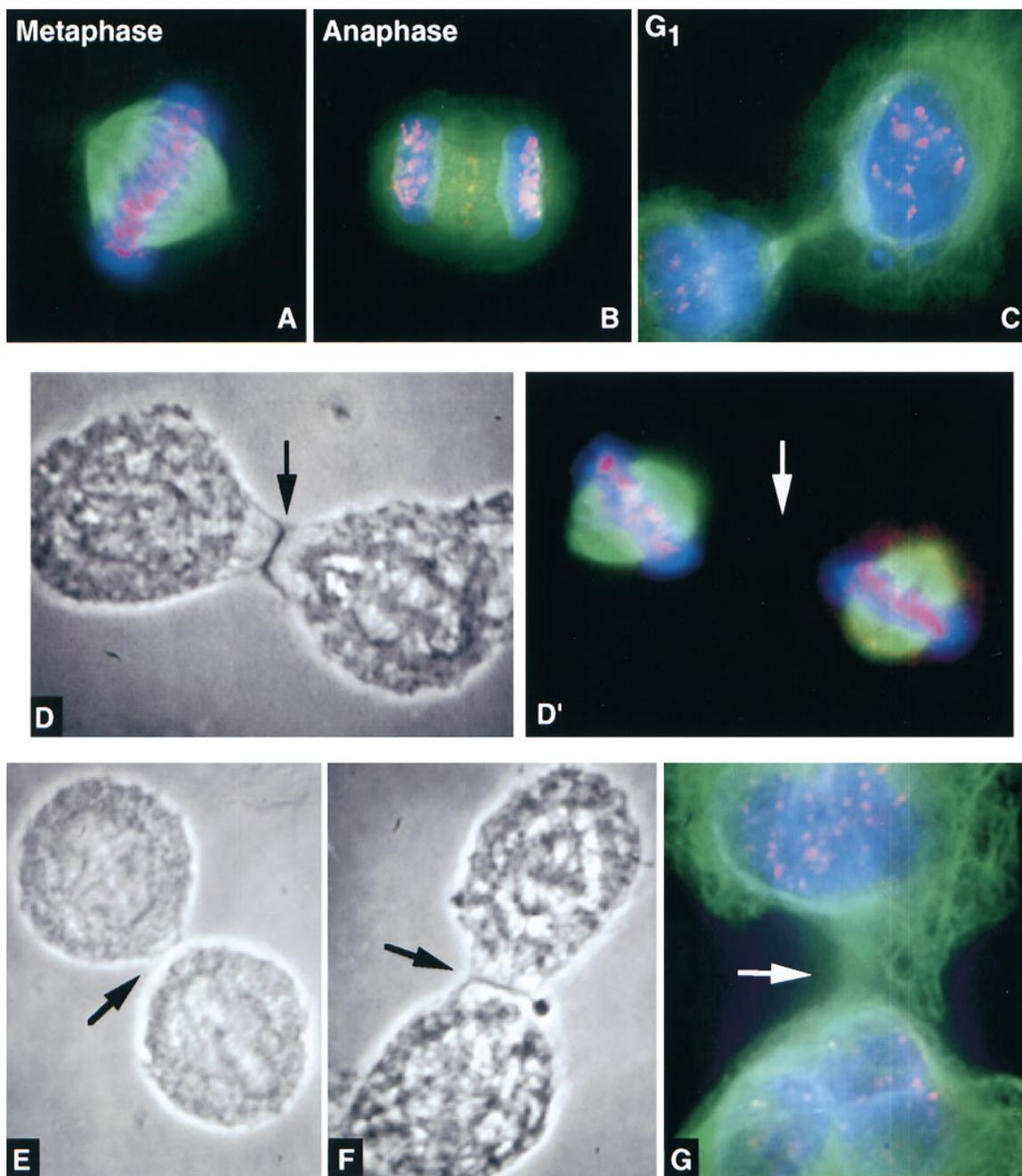


Figure 9. A dominant negative INCENP mutant disrupts the completion of cytokinesis. (A–C) Association of CENP-B₁₋₁₅₈:INCENP₄₃₋₈₃₉ with centromeres during mitosis. In these images of transfected cells, taken 24 h after transfection, CENP-B₁₋₁₅₈:INCENP₄₃₋₈₃₉ is red, DNA is blue, and microtubules are green. The chimeric protein remains associated with centromeres at all stages of the mitotic cycle. (D and D') Paired metaphases observed 48 h after transfection. In the phase contrast image (D), two cells are seen to be joined by a prominent intracellular bridge with a midbody (arrow). In the immunofluorescence image (D'), both cells are seen to be in metaphase of the next cell cycle. Neither tubulin nor INCENPs are detected in the midbody. (E and F) Two further examples of paired metaphase cells joined by prominent intercellular bridges with midbodies. (G) Dikaryon with a prominent intercellular bridge containing an interphase array of microtubules.

Discussion

Adjacent Spindle Poles Do Not Necessarily Trigger Cleavage Furrow Formation

Classic micromanipulation studies using echinoderm eggs (Rappaport, 1961) led to the now widely established “as-

tral stimulation” model, which states that cleavage furrows form midway between adjacent spindle asters, regardless of whether or not they are linked by a differentiated central spindle with attached chromosomes. This model was also supported by a more recent study in which pressure treatment was used to produce eggs with two spindles

(Salmon and Wolniak, 1990). Furthermore, the notion that spindle poles, rather than chromosomes, are important for cleavage furrow assembly is supported by microsurgery studies in which removal of nuclei (Sluder et al., 1986) or meiotic chromosomes (Zhang and Nicklas, 1995b; Zhang and Nicklas, 1996) from cells did not block the normal initiation of furrowing.

The above experiments were performed in specialized cell types: either fertilized eggs or spermatocytes. We therefore wanted to test whether the “astral stimulation” model would apply equally well to dividing mammalian somatic cells. We devised a protocol that enabled us to fuse adjacent human U2OS mitotic cells without the use of treatments that perturb the mitotic spindle or cleavage furrow. This cell fusion produces heterokaryons with two spindles aligned in a V configuration. At metaphase, the chromosomal mass appears to fuse laterally into a single bent metaphase plate. This configuration is roughly similar to that obtained with pressure treatment of eggs (Salmon and Wolniak, 1990). However, we obtained a dramatically different result upon observation of the progress of the heterokaryons through cytokinesis. In 15 of 19 cases, we observed a single cleavage furrow located above the region previously occupied by the metaphase plate. In 4 of 19 heterokaryons, we observed the formation of a second, fully functional, ectopic furrow between the adjacent spindle poles at the open end of the V. The existence of this minority class confirms that furrows can form above microtubule arrays lacking chromosomes, even in vertebrate cultured cells. These results, together with recent work using rodent cells (Wheatley and Wang, 1996), suggest that “astral stimulation” does not appear to be the sole mechanism for cleavage furrow positioning in mammalian cells.

Why are different results obtained with somatic cells and early embryos? One obvious difference between the two cell types is that the latter contain stockpiles of components needed for replication and division. For example, *Xenopus* eggs contain stockpiles of soluble INCENP that are not associated with chromosomes (Eckley, D.M., unpublished observations). In contrast, somatic cells do not appear to have surplus INCENP (Cooke et al., 1987). The presence of stockpiles of INCENP and/or other components needed for furrow assembly or function in embryos might favor the formation of ectopic furrows when the normal cellular geometry is perturbed. It will be important to repeat the classical micromanipulation experiments once cross-reactive antibody probes are available, to assess the distribution of furrow components and to determine whether perturbation of their function (by antibody microinjection) influences the furrowing process. It is worth noting that this is not the only instance of different results being obtained when comparing mitotic events in early embryos and cultured somatic cells. For example, studies of the “wait anaphase” checkpoint reveal that maloriented chromosomes do not stop the metaphase–anaphase transition in invertebrate embryos, while they do effectively block this transition in PtK cells (Sluder et al., 1994; Rieder et al., 1995).

The experiments that lead to the astral stimulation model of cytokinesis provide no hint as to the identity of the critical molecules that are responsible for directing the assembly and function of the cleavage furrow. However,

our previous immunolocalization studies had suggested that INCENP and other chromosome passenger proteins could have a role in these processes.

A Role for the Spindle Midzone in Cytokinesis: Are Chromosomal Passengers Involved?

Studies with both early embryos and cultured cells lead to the conclusion that the spindle midzone has a role in cleavage furrow formation and function. In a classic study of the nature of the cleavage stimulus, a physical barrier (a tear) was introduced between the central spindle and the cortex of an echinoderm embryo (Rappaport and Rappaport, 1983). At the time of furrow initiation, normal furrowing was observed at the inner surface of the tear, but there was no furrowing of the outer cortex. This led to the conclusion that the cleavage stimulus travels outward in a straight line from the central spindle to the cortex. Recently, this study was repeated using cultured rat kidney cells. Placement of a perforation between the central spindle and the cortex before anaphase onset was found to abolish furrowing of the cortex and also to prevent formation of a normal ordered microtubule array in the cytoplasm between the perforation and the cortex (Cao and Wang, 1996). As in the echinoderm embryos, a furrow appeared to form at the inner surface of the perforation. Although this furrow functioned normally, cytokinesis was incomplete since the cytoplasm distal to the perforation failed to cleave. Inhibition of cleavage was only observed if the perforation was located on a line bisecting the location of the metaphase plate. Thus, it was concluded that the signal to the cortex must be released from a relatively localized region near the spindle midzone and travel laterally from there to the cell cortex (Cao and Wang, 1996). Release of this signal (from either the chromosomes or interpolar microtubules) appeared to occur at about the time of anaphase onset; if the cell was perforated after anaphase onset, normal furrowing occurred all across the equator, and the cell divided normally.

Electron microscopy of anaphase cells reveals that the bundled antiparallel microtubules of the spindle midzone are coated by electron-dense material, called stem body matrix (Buck and Tisdale, 1962). The composition of this material and its biochemical role remain unknown. Based on our immunoelectron microscopy studies of INCENP (Earnshaw and Cooke, 1991) and CENP-E (Cooke, C.A., and W.C. Earnshaw, unpublished results), we suggest that chromosomal passenger proteins may comprise a significant percentage of the stem body material.

Chromosomal passengers are proteins that are tightly bound to chromosomes during the early stages of the cell cycle (and often throughout the entire interphase portion of the cell cycle) but that associate with the spindle midzone and (in some cases) cleavage furrow during anaphase (Earnshaw and Bernat, 1990). We have earlier proposed that the passengers may be specialized mitotic cytoskeletal proteins that rely upon the movements of the chromosomes for their exact positioning during mitosis (Earnshaw and Bernat, 1990). An involvement in spindle structure and cytokinesis has been demonstrated for one chromosomal passenger, the kinesin-like protein variously known as KLP3A, XKLP1, KIF4, and chromokinesin (Sekine et al.,

1994; Vernos et al., 1995; Wang and Adler, 1995; Williams et al., 1995). When XKLP1 mRNA levels were decreased in frog egg extracts by antisense RNA treatment, spindle assembly was defective, and treated fertilized eggs failed to divide (Vernos et al., 1995). Similarly, in KLP3A mutant flies (Williams et al., 1995), testis cells failed to form organized central spindles and became multinucleate as a result of subsequent failures in cytokinesis. While it is not particularly surprising that a kinesin-related protein has a function in spindle structure, the role of other chromosomal passenger proteins such as INCENP, which does not exhibit structural similarity to known microtubule motor proteins, is less obvious.

Evidence for the Involvement of INCENP in Cytokinesis

Evidence from this and our previous studies is consistent with INCENP having a role in either the assembly or function of the cleavage furrow. Thus, INCENP is located in the right place within the cell, it locates there at the right time (early in the assembly of the presumptive furrow), and its distribution follows the distribution of ectopic furrows when the geometry of cytokinesis is perturbed. Furthermore, expression of a dominant mutant form of INCENP is able to disrupt the completion of cytokinesis.

In the original description of INCENP localization in dividing cells, it was observed that in addition to its localization on the central spindle, the protein was also concentrated at the cell cortex in the cleavage furrow (Cooke et al., 1987). Subsequent immunoelectron microscopy studies using 1-nm colloidal gold revealed that a subpopulation of INCENP in cells undergoing cytokinesis is concentrated in the cleavage furrow, on the inner surface of the plasma membrane (Earnshaw and Cooke, 1991). Thus, INCENP colocalizes with the contractile ring in dividing cells.

INCENP localizes to the equatorial cortex relatively early in anaphase. We have shown previously that INCENP concentrates in this region before any sign of furrowing (Earnshaw and Cooke, 1991). The present study reveals that INCENP, myosin, and radixin accumulate in the presumptive furrow in a reproducible sequence. Surprisingly, the chromosomal protein precedes the two actin-binding proteins to the furrow.

If INCENP does have an important role in cleavage furrow assembly or function, then physical alterations of the spindle architecture that induce ectopic furrow formation should also induce changes in INCENP distributions. We have confirmed this hypothesis by examining the INCENP distribution in the mitotic heterokaryons described above and in rare sporadic failures in cytokinesis found in untreated cultures. In both cases, INCENP was concentrated at all sites where furrowing was taking place and was not found at analogous sites in other cells where furrowing was not occurring. A similar correlation has recently been described for the chromosomal passenger TD-60 (Wheatley and Wang, 1996).

Both evidence presented here and studies to be reported elsewhere (Mackay, A.M., and W.C. Earnshaw, unpublished observation) provide a functional link between INCENP and cytokinesis. Here we have shown that a chimeric CENP-B:INCENP fusion protein that is tethered at

centromeres produces a dominant phenotype in dividing cells. This phenotype is characterized by a failure of the cells to complete cytokinesis: many transfected cells apparently arrest part way through cytokinesis with an established midbody structure but then enter the next cell cycle without completing cleavage. In other studies, we have shown that an INCENP half-molecule that is missing the microtubule-binding domain also remains tethered on chromosomes throughout mitosis (Mackay, A.M., and W.C. Earnshaw, unpublished observation). This dominant mutant INCENP produces a range of phenotypes in mitotic cells, but one that is observed frequently is a failure to complete cytokinesis: cells begin to divide, but once the furrow has constricted about half-way, furrowing is reversed and the cell exits mitosis as a binucleate heterokaryon (Mackay, A.M., and W.C. Earnshaw, unpublished observation). The explanation for the failure in cytokinesis induced by the INCENP mutants is not yet known. The simplest interpretation of these experiments is that INCENPs either participate directly in cytokinesis or interact with other components of the cleavage machinery.

A recent study showing that surgical removal of chromosomes from bipolar spindles of grasshopper spermatocytes did not block the onset of anaphase or cytokinesis appears to argue against the notion that chromosomal proteins would have a role in cleavage furrow formation or function (Zhang and Nicklas, 1996). An earlier study from the same group demonstrated that chromosomal proteins are necessary for the assembly of bipolar spindles in these cells (Zhang and Nicklas, 1995a). Together, these experiments suggest that chromosomal proteins are necessary for the assembly of the bipolar spindle (Zhang and Nicklas, 1995a) but may not be required once spindle assembly is complete. However, two caveats must be considered when interpreting these studies: (a) Cytokinesis during spermatogenesis does not necessarily go to completion (Dym and Fawcett, 1971). It is therefore possible that the cleavage mechanism in spermatocytes may differ in detail from that in other somatic cells. (b) The authors did not perform a biochemical or immunochemical analysis on the cells after microsurgery. It is possible that during removal of the chromosomes, some chromosomal passenger proteins were detached and remained associated with the spindle. Furthermore, in these cells some or all passenger proteins may be released from the chromosomes at the time of bipolar spindle formation. Thus, although this is clearly an important study, further biochemical analysis is needed before its implications for the role of chromosomal proteins in cleavage furrow assembly and function will be fully understood.

One possible role for INCENP and/or other chromosome passenger proteins might be to position either myosin or actin filaments in the presumptive cleavage furrow (Margolis and Andreassen, 1993). INCENP might bind directly to myosin molecules or filaments, recruiting them directly to the cell equator, so they can participate in contractile ring function. Alternatively, INCENP and/or other chromosome passenger proteins might position the actomyosin contractile apparatus without binding directly to it. The stem body matrix has been observed at the cleavage furrow in several electron microscopy studies of dividing cells (Schroeder, 1972; Zeligs and Wollman, 1979, 1981;

Maupin and Pollard, 1986). Microtubules associated with INCENP and other components of the stem body material form an equatorial ring structure before myosin accumulation is detected. This ring might conceivably act as a template for formation of an actomyosin contractile ring. Actin filaments are recruited to the cell equator by cortical flow during anaphase (Koppel et al., 1982; Bray and White, 1988; Cao and Wang, 1990). The bundled microtubules of the stem body material might position microfilaments at the cleavage furrow by perturbing this cortical flow at the cell equator. Actin filaments from both ends of the cell might then accumulate at the presumptive cleavage furrow, as observed by Fishkind and Wang (1993).

Clearly, it will be important to further characterize the stem body material to confirm whether it does play an active role in cleavage furrow assembly, and if so, to identify the relevant functional constituents and the mechanism of their transport from the chromosome to the furrow.

We thank Abigail and Gary Conrad for the gift of myosin antibody; S. Tsukita for the gift of anti-radixin antibody; T.D. Pollard, T. Schroer, K. Wilson, S.A. Krause, and M.M.S. Heck for helpful editorial suggestions, and other members of the Earnshaw lab for critical reading of the manuscript.

These studies were supported by National Institutes of Health grant GM35212 in Baltimore and a Wellcome Principal Research Fellowship in Edinburgh.

Received for publication 25 September 1996 and in revised form 8 January 1997.

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