A Dominant Mutant of Inner Centromere Protein (INCENP), a Chromosomal Protein, Disrupts Prometaphase Congression and Cytokinesis

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Abstract. INCENP is a tightly bound chromosomal protein that transfers to the spindle midzone at the metaphase/anaphase transition. Here, we show that an INCENP truncation mutant (INCENP_{382-839}) associates with microtubules but does not bind to chromosomes, and coats the entire spindle throughout mitosis. Furthermore, an INCENP truncation mutant (INCENP_{43-839}) previously shown not to transfer to the spindle at anaphase (Mackay, A.M., D.M. Eckley, C. Chue, and W.C. Earnshaw. 1993. J. Cell Biol. 123:373–385), is shown here to bind chromosomes, but is unable to target to the centromere. Thus, association with the chromosomes, and specifically with centromeres, appears to be essential for INCENP targeting to the correct spindle subdomain at anaphase. An INCENP truncation mutant (INCENP_{1-405}) that targets to centromeres but lacks the microtubule association region acquires strong dominant-negative characteristics. INCENP_{1-405} interferes with both prometaphase chromosome alignment and the completion of cytokinesis. INCENP_{1-405} apparently exerts its effect by displacing the endogenous protein from centromeres. These experiments provide evidence of an unexpected link between this chromosomal protein and cytokinesis, and suggest that one function of INCENP may be to integrate the chromosomal and cytoskeletal events of mitosis.

Although it is now clear that chromosomes are not simply passive objects that are acted upon by the cytoskeleton during mitosis, the extent of their contribution to cytoskeletal events such as cytokinesis remains controversial. At one extreme, it has been suggested that chromosomes are completely dispensable for mitotic events once a bipolar spindle has been assembled (Zhang and Nicklas, 1996). However, other studies suggest that although nuclei are not needed for the initiation of cytokinesis, they are required for cleavage furrows to progress to completion (Sluder et al., 1986; Rappaport, 1991). It now appears that cytokinesis requires a pair of complementary stimuli; one initiating furrow formation, and the other allowing the process to go to completion (Rappaport, 1991). The former is likely to be cytoskeletal in origin, whereas the latter may originate on or near the chromatin. How and where these factors act is not clear, and in particular, the role of the spindle midzone in promoting furrowing is presently controversial. On one hand, certain studies suggest that an ordered spindle midzone with segregating chromosomes has an essential role in the stimulation of furrowing in somatic cells (Cao and Wang, 1996; Wheatley and Wang, 1996). However, other studies suggest that furrows can form midway between asters that are not connected by an ordered midzone (Eckley et al., 1997; Rieder et al., 1997).

One candidate for a nuclear factor involved in stabilizing cleavage furrows is inner centromere protein (INCENP)\(^1\), a “chromosomal passenger” protein (Cooke et al., 1987; Earnshaw and Bernat, 1990). Passengers are tightly associated with chromosomes in the early stages of mitosis, and many of these proteins transiently concentrate at centromeres during metaphase (Earnshaw and Mackay, 1994). Passengers abruptly dissociate from the chromosomes at the metaphase/anaphase transition and associate with the stem body material coating the overlapping antiparallel microtubules of the central spindle (Buck and Tisdale, 1962). During anaphase, a portion of INCENP also concentrates in the presumptive cleavage furrow before myosin II or radixin and before any hint of furrowing, making it one of the earliest known markers for furrow assembly (Eckley et al., 1997). A second passenger protein, TD-60, is also an early marker of furrow assembly (Martineau et al., 1987).

1. Abbreviations used in this paper: TD-60, telophase disk protein of 60 kD; DAPI, 4,6-diamidino-2-phenylindole; GFP, green fluorescent protein; INCENP, inner centromere protein; CENP-E, centromere protein E.
were analyzed 18 h after electroporation.

10% fetal bovine serum. Except where noted, transiently transfected cells

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Microcolonies of 16–32 LLCPK or HeLa cells on gridded coverslips were

trypsinized, rinsed pig (LLCPK) cells resuspended in 0.4 ml Opti-MEM I

Buffers and Reagents

Buffers is 10 mM Tris-HCl, pH 7.4

KA buffer is 7.5 mM Tris-HCl, pH 7.4, 40 mM KCl, 1.0 mM K-EDTA, pH 7.4,

KB buffer is 10 mM Tris-HCl, pH 7.7, 150 mM NaCl, 0.1% BSA. A/2

Buffers and Reagents

Electroporation of Cells

Electroporation of Cells

Materials and Methods

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truncation mutants to map INCENP regions that are essential for centromere targeting and transfer to the spindle during anaphase. The constructs and results are summarized in Fig. 1. In transfected cells expressing INCENP at extremely high levels (“jackpot” cells), INCENP accumulates in the cytoplasm, where it colocalizes with microtubules (Mackay et al., 1993) and causes a dramatic change in the organization of the microtubule network (Fig. 2 A). Based on this assay for microtubule association, we have found that the transfer of INCENP from the chromosomes to the mitotic spindle is unexpectedly complex: an INCENP truncation mutant (INCENP<sub>382–839</sub>) that fails to associate with microtubules in the interphase cytoplasm (Mackay et al., 1993) does transfer to the spindle, whereas another mutant (INCENP<sub>43–405</sub>) that can associate with microtubules (Fig. 2 B) is defective in spindle transfer (Mackay et al., 1993).

If microtubule association is not the key to spindle association, then what is? Most chromosome passenger proteins become highly concentrated at centromeres before anaphase onset (Earnshaw and Mackay, 1994). Therefore, it is possible that centromere targeting in metaphase is required for spindle transfer in anaphase. To test this possibility, we have examined the distribution of INCENP in mitotic chromosomes obtained by centrifuging colcemid-arrested cells onto coverslips. This experiment enabled us to visualize the localization of both endogenous porcine and transfected wild-type chicken INCENP in the inner centromere, flanked by a marker for the kinetochore domain (Fig. 3, A and B). In striking contrast, INCENP<sub>43–839</sub> was found to be defective in centromere targeting, instead painting the entire chromosome (Fig. 3 D). Two other INCENP truncation mutants (Fig. 1, INCENP<sub>351–696</sub>, and INCENP<sub>1–598</sub>) that are defective in microtubule binding but positive for spindle transfer, both showed strong centromere targeting (Fig. 3 C; data not shown). Thus, the amino-terminal 42 amino acids of INCENP are essential for centromere targeting, and this targeting correlates with the ability to transfer to the spindle at anaphase.

To test the hypothesis that centromere targeting alone is necessary and sufficient for INCENP to transfer to the spindle at anaphase onset, we constructed an INCENP truncation mutant (Fig. 1, INCENP<sub>43–405</sub>) that showed strong targeting to centromeres (Fig. 3 E), and no detectable association with microtubules in the cytoplasm of jackpot cells (Fig. 2 C). In selected cells where the INCENP<sub>43–405</sub> was expressed at nearly endogenous levels and no obvious perturbation of mitotic events was observed, INCENP<sub>43–405</sub> localization resembled that of wild-type INCENP in early mitosis through metaphase (Fig. 4, A–B'). However, after anaphase onset, when the endogenous INCENP transferred to the spindle (Fig. 4 C), INCENP<sub>43–405</sub> remained on the separating sister chromatids (Fig. 4 C'). Low levels of INCENP<sub>43–405</sub> could be detected in the intercellular bridge of cells undergoing cytokinesis, although most of the transfected protein remained in the daughter nuclei (Fig. 4, compare D and D').

Identification of a Domain Required for INCENP Targeting to the Spindle Midzone

If INCENP is capable of associating with microtubules along their entire lengths in jackpot cells, how is it that the protein targets selectively to the spindle midzone at anaphase, rather than binding throughout the mitotic spindle? The preceding experiments, together with those of an earlier study (Mackay et al., 1993), confirmed that the carboxy-terminal half of INCENP is required for the protein to associate with microtubules in the cytoplasm of jackpot cells. To determine whether the microtubule association region of INCENP contains a spindle midzone–targeting motif, we created a truncated INCENP molecule, INCENP<sub>382–839</sub>, that contains the microtubule association region, but lacks the amino-terminal half of the protein (Figs. 1 and 5).

This molecule accumulated in nuclei during interphase (Fig. 5 A). However, as cells entered mitosis, the protein showed no association with the condensing chromosomes. After nuclear envelope breakdown, INCENP<sub>382–839</sub> appeared to coat the entire spindle, so that by metaphase (Fig. 5 B), the INCENP staining essentially duplicated that seen with an antitubulin antibody. After anaphase onset, INCENP<sub>382–839</sub> showed no preferential association with the central spindle. Instead it continued to coat the spindle microtubules nondiscriminately (Fig. 5 C). These experiments indicate that the microtubule association region of INCENP...
lacks a specific spindle midzone–targeting signal. Thus, it appears that some activity directed by the amino-terminal portion of INCENP, possibly association with chromosomes or targeting to centromeres, is essential for the correct localization of INCENP to the midzone at anaphase.

**INCENP<sup>1–405</sup> Is a Dominant Mutant That Interferes with Cell Proliferation**

Use of a quantitative colony counting assay (Bernat et al., 1990) revealed that the expression of INCENP<sub>1–405</sub> inhibited the proliferation of human (HeLa) and pig (LLCPK) cells. In the experiment shown in Fig. 6 A, HeLa cells were plated sparsely and then allowed to grow into microcolonies. At time \( t = 0 \), all cells in selected colonies were microinjected with an expression construct encoding INCENP<sub>1–405</sub>. By 36 h later the number of cells per colony remained slightly below that at the start of the experiment. Under these conditions, neighboring colonies of un.injected cells grew with a doubling time of \( \sim 25 \) h. Cells injected with a construct expressing INCENP<sub>43–839</sub> showed an initial growth lag, but had nearly doubled by 36 h. Similar results were obtained after injection of full-length wild-type INCENP, INCENP<sub>382–839</sub> or INCENP<sub>43–405</sub> (data not shown).

**INCENP<sup>1–405</sup> Interferes with Prometaphase Chromosome Alignment**

Transfected cultures expressing INCENP<sub>1–405</sub> exhibited an increased percentage of cells in prometaphase and ana/telophase relative to control transfectants, while showing a concomitant decrease in the number of cells in metaphase and undergoing normal cytokinesis (Fig. 6 B). Control cultures transfected with the pECE vector alone or with INCENP<sub>43–405</sub> showed essentially identical levels of cells throughout the various mitotic stages (Fig. 6 B). Thus, INCENP<sub>1–405</sub> appears to interfere with both chromosome congression in prometaphase and with events during ana/telophase.

To examine events during prometaphase in more detail, cultures of HeLa and LLCPK cells were transfected with vector alone or with constructs encoding INCENP<sub>1–405</sub> or, as a control, INCENP<sub>43–405</sub>. Transfected cultures were then scored for the percentages of cells in the various mitotic phases.
stages. Relative to the control transfectants, expression of INCENP1-405 had no effect on the frequency of cells with no organized plate (Figs. 6 C and 7 A), nor with up to three mono-oriented chromosomes. Since the percentage of cells in a particular mitotic phase is proportional to the length of that phase, we conclude that expression of INCENP1-405 does not result in a significant delay in the initiation of congression.

In contrast, a substantial increase (\( \sim 25\% \)) was noted in the number of cells expressing INCENP1-405 that had a metaphase plate but with at least four maloriented chromosomes (Figs. 6 C and 7 B). The simplest interpretation of this result is that some cells expressing INCENP1-405 arrest or delay in midprometaphase (the hypercondensation of the chromosomes in Fig. 7 B is consistent with a mitotic delay). Observation of the INCENP signal in indirect immunofluorescence suggests that the more severely affected cells express elevated levels of INCENP relative to the endogenous protein.

The accumulation of cells expressing INCENP1-405 with at least four chromosomes not aligned at the metaphase plate could arise either from a failure of a subset of chromosomes to congress to the spindle midzone or from instability of the plate once congression had occurred. As an initial approach to distinguish between these two alternatives, we fused INCENP1-405 to the Aequorea victoria GFP (Chalfie et al., 1994). Among cells expressing the INCENP1-405–GFP chimera, we could observe cells in which congression appeared to be delayed (data not shown). Thus, these preliminary results provide support for the notion that the expression of INCENP1-405 can interfere with the completion of prometaphase congression in living cells.

The apparent interference with prometaphase congression by INCENP1-405 does not appear to result in a complete block of mitotic progression. As described below, many cells expressing INCENP1-405 were observed in various stages of anaphase. As these cells often showed the presence of lagging chromosomes or micronuclei, we deduce that, as described previously in other cell types (see Molé-Bajer, 1958; Sluder, 1979; Rieder and Alexander, 1989; Sluder et al., 1994 for selected examples), mitotic checkpoint control in these cells was not sufficient to completely block mitotic progression in the presence of the mitotic abnormalities caused by expression of INCENP1-405.
Figure 4. Distribution of endogenous INCENP, INCENP1–405 and INCENP43–405 in LLCPK cells undergoing normal mitosis. Control cells (A–D) or cells transfected with either INCENP1–405 (A’–D’) or INCENP43–405 (B’’–D’’) were stained for INCENP (red), microtubules (green) and DNA (blue). (A and A’) Interphase through prophase: endogenous INCENPs are nuclear. (B and B’) Prometaphase through metaphase: both endogenous INCENPs and INCENP1–405 become highly concentrated at centromeres (arrow). INCENP43–405 is distributed all along the chromosomes with no enrichment at centromeres (arrow). (C and C’) Anaphase: endogenous INCENP transfers to the central spindle and cell cortex in the cleavage furrow. INCENP1–405 and INCENP43–405 remain on the separated sister chromatids. (D and D’) Cytokinesis: endogenous INCENP labels the intercellular bridge intensely; nuclei are unlabeled. INCENP1–405 is found in both daughter nuclei and the intercellular bridge. INCENP43–405 is found in daughter nuclei and is absent from the intercellular bridge. Although quantitation of expression levels in transient transfections is problematic, it is our impression that cells expressing low levels of INCENP1–405 traverse mitosis normally, whereas cells expressing higher levels of the mutant protein frequently exhibit disruptions of mitosis. Bar, 10 μm.
INCENP<sub>1-405</sub> Disrupts Cytokinesis

Colonies of microinjected HeLa cells expressing INCENP<sub>1-405</sub> contained, on average, 46% binucleate cells by 36 h after injection, strongly suggesting that this protein can interfere with cytokinesis. This prediction was verified by direct examination of living cells (Fig. 8). Microinjected HeLa cells expressing INCENP<sub>1-405</sub> traversed mitosis and furrowing was apparently initiated normally. At that point, a number of cells appeared to stall in their progression through cytokinesis. For example, the cell in Fig. 8 B appeared to pause with the furrow constricted about halfway for 12 min before abruptly returning to a rounded configuration. No such disruptions of cytokinesis were observed in cells expressing INCENP<sub>43-405</sub> or INCENP<sub>43-839</sub> (data not shown). We note that this disruption of cytokinesis was observed in both human (HeLa) and pig (LLCPK) cells expressing INCENP<sub>1-405</sub>.

Examination of fixed samples revealed the presence of two classes of abnormal ana/telophase cells expressing INCENP<sub>1-405</sub> or INCENP<sub>43-839</sub> (data not shown). We note that this disruption of cytokinesis was observed in both human (HeLa) and pig (LLCPK) cells expressing INCENP<sub>1-405</sub>.

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To rule out the possibility that expression of INCENP<sub>1-405</sub> somehow causes the endogenous INCENP to be degraded, HeLa cells were electroporated with constructs expressing INCENP<sub>1-405</sub> or INCENP<sub>43-405</sub> together with a construct expressing a Golgi marker protein (human ELPI) conjugated to GFP (Cole et al., 1996) and sorted by FACSTM 18 h later, yielding a population of transfected
INCENP1–405 Is a Dominant Mutant That Disrupts Prometaphase Congregation

INCENP1–405 binds to chromosomes and targets to centromeres but lacks the motifs required for association with microtubules in jackpot cells. In mitosis, this protein localizes normally from prophase through metaphase, but from anaphase onward it fails to transfer to the spindle, instead remaining bound to the chromosomes. If expressed at elevated levels, INCENP1–405 is toxic to dividing cells, causing defects both early and late in mitosis.

The earliest mitotic defect seen in cells expressing INCENP1–405 is a failure to complete prometaphase chromosome alignment. Approximately 40% of cells expressing INCENP1–405 appear to arrest or delay in midprometaphase with at least four mono-oriented chromosomes. In theory, this phenotype could arise either by interference with the movement of the chromosomes to the spindle midzone (congression defect), or by destabilization of the metaphase configuration itself: chromosomes might congress, but then subsequently detach and become randomized in the cell. We have previously observed the latter phenotype in cells injected with anticentromere antibodies that interfere with kinetochore structure and stability (Bernat et al., 1990, 1991). In the case of INCENP1–405, we believe that the most likely defect is in congression itself, as we have directly observed a significant delay in chromosome congression in living cells expressing an INCENP1–405–GFP fusion protein (data not shown).

INCENP1–405 might disrupt congression by interfering with the assembly of a chromosomal structure such as the kinetochore. To investigate this possibility, cells microinjected with plasmid encoding INCENP1–405 were monitored by video microscopy. Selected cells undergoing difficulties in mitosis were fixed, embedded, and then subjected to serial sectioning analysis in the electron microscope. No significant changes in kinetochore ultrastructure were observed (Cooke, C.A., A. Mackay, and W.C. Earnshaw, unpublished observations; data not shown).

Alternatively, INCENP1–405 might interfere with the function of plus end-directed motors on the chromosomes such as chromokinesin–Xklp-1 (Vernos et al., 1995; Wang and Adler, 1995), mitotic centromere-associated kinesin (Wordeman and Mitchison, 1995), or centromere protein E (CENP-E) (Yen et al., 1991, 1992; Wood et al., 1997). Interference with CENP-E function either in vitro (Wood et al., 1997) or in vivo (Schaar et al., 1997) has been shown to disrupt prometaphase chromosome alignment. Interestingly, microinjection of antibodies to CENP-E into cultured cells produced an effect very similar to that seen after expression of INCENP1–405: prometaphase cells accumulated in which a limited subset of the chromosomes failed to congress to the metaphase plate (Schaar et al., 1997). Those authors suggested that chromosomes near poles encounter fewer microtubules and therefore, might require more effi-
cient capture mechanisms (presumably involving CENP-E) to congress properly. It will be interesting in future experiments to determine whether expression of INCENP1–405 alters the distribution of CENP-E in mitotic cells.

Finally, it is possible that expression of INCENP1–405 interferes with some other aspect of spindle assembly or function that is required for cells to attain a stable metaphase configuration. In particular, INCENP1–405 might interfere with the function of the stem body matrix, a poorly characterized electron-dense material that coats the antiparallel microtubules of the central spindle (Buck and Tisdale, 1962). Although the stem body matrix is generally thought to function in anaphase, it is possible that this structure has a role earlier in mitosis, e.g., in stabilization of the spindle during formation of the metaphase plate. Immuno-electron microscopy reveals that INCENP can transfer from the chromosomes to the stem body matrix during metaphase (Earnshaw and Cooke, 1991). It is worth noting that three proteins required for prometaphase congression, chromokinesin–Xklp-1 (Vernos et al., 1995), CENP-E (Schaar et al., 1997; Wood et al., 1997), and INCENP (this study) all associate with the stem body matrix later in mitosis.

**Disruption of Cytokinesis by INCENP1–405**

The second phenotype commonly seen in cells expressing
elevated levels of INCENP_{1-405} was a failure to complete cytokinesis: cleavage furrows constricted to a variable extent, usually approximately halfway, but then abruptly regressed, causing the cell to return to a rounded state. One explanation for such a failure in cytokinesis could be that the cleavage furrow impinges upon lagging chromosomes and is physically prevented from going to completion. In a careful light and electron microscopy study of the terminal phase of cytokinesis (Mullins and Biesele, 1977), it was noted that the presence of chromatin in the intercellular bridge caused cytokinesis to fail, followed by furrow regression and the production of binucleate cells. We have produced an identical phenotype in previous studies from our lab by injecting cells with anticentromere antibodies from patients (Bernat et al., 1990, 1991). We do not believe this effect can explain the present results for two reasons. First, in all cases where chromatin was trapped in the midbody, cytokinesis appeared to proceed normally until it encountered the lagging chromosome. This resulted in the appearance of a highly constricted intercellular bridge with a well-formed midbody containing trapped chromatin (Mullins and Biesele, 1977). This midbody persisted after

**Figure 8.** Examples of failures in cytokinesis in cells expressing INCENP_{1-405}. (A and B) Examples of two HeLa cells injected with a plasmid encoding INCENP_{1-405} at time $t = 0$. Observation began 15 h, 40 min after microinjection. Both cells show indications of furrowing that ultimately reverse.

**Figure 9.** Expression of INCENP_{1-405}, but not INCENP_{43-405}, displaces the endogenous INCENP from its normal location. Endogenous INCENP detected using a polyclonal antibody specific for INCENP residues 404-839 is diffusely distributed in transfected cells expressing INCENP_{1-405} either during prometaphase (A, A', and A''), anaphase (B, B', and B'') or telophase (C, C', and C''). In contrast, the distribution of endogenous INCENP is normal in cells expressing INCENP_{43-405} (D, D', and D''). Staining for the endogenous protein is seen in A–D, with the expected location of INCENP indicated by an arrow in each panel. The expressed truncated INCENPs are seen in A–D', A''–D''. A''–D'' show the DAPI staining for DNA. Similar results were obtained with cells at all stages of the cell cycle. Bar, 10 μm.
regression of the furrow. In the present study, cytokinesis apparently failed at a much earlier stage; no fully constricted intercellular bridges were noted, and no midbody remnants were found in binucleate cells produced as a consequence of INCENP1–405 expression. In the earlier study (Mullins and Biesele, 1977), observation of living cells revealed that the stalled intercellular bridge did not regress for 1–9 h. In the present study, furrow regression appeared to occur much more rapidly (in as little as 7 min with an average of 21 ± 16 min in 9 cells followed by video microscopy). Finally, as described below, we have previously shown that a chimeric CENP-B–INCENP45–839 fusion protein also blocks the completion of cytokinesis in human cells (Eckley et al., 1997). Cells expressing that protein showed no obvious disruption of prometaphase events nor evidence of lagging chromosomes.

A number of earlier observations support the suggestion that INCENP might have a role in cytokinesis. (a) INCENP is one of the earliest known polypeptides to concentrate in the presumptive cleavage furrow; the protein is detected in this region during midanaphase before any evidence of furrowing (Cooke et al., 1987), and before the accumulation of myosin II and radixin (Eckley et al., 1997). (b) Immunoelectron microscopy reveals that in addition to its association with the stem body matrix, a portion of the INCENP antigen is intimately associated with the cytoplasmic face of the plasma membrane within the cleavage furrow (Earnshaw and Cooke, 1991). (c) In cells undergoing aberrant multipolar cytokinesis, INCENP is always associated with sites of both normal and ectopic furrowing (Eckley et al., 1997). (d) Expression of an artificial protein in which INCENP45–839 was fused to the centromere-targeting motif from CENP-B, a DNA-binding protein that is localized to the same region of the centromeric heterochromatin as INCENP (Cooke et al., 1987; Earnshaw and Cooke, 1991), caused a dominant failure late in cytokinesis (Eckley et al., 1997). Importantly, the terminal phenotype produced by the artificial chimera was distinct from that produced by INCENP1–405: Cleavage failed at a later stage, the furrow did not regress, and cells proceeded into the next cycle joined by an intercellular bridge with a prominent midbody (Eckley et al., 1997). Thus, two different dominant INCENP mutants, which target to centromeres by distinct mechanisms, both interfere with the completion of cytokinesis but produce distinct terminal phenotypes. The fact that the disruption of cytokinesis is not an allele-specific phenotype lends strong support to the notion that INCENP or an interacting protein has an important role in cytokinesis.

Centromere Targeting Is Essential for INCENP Function

We have identified an INCENP region that is essential for centromere targeting and shown that this targeting is crucial for the function of both the wild-type INCENP and its dominant mutant alleles. In fact, the best explanation for the dominant-negative effect of INCENP1–405 is that this protein displaces the wild-type endogenous protein from its centromeric binding sites. This implies that the centromeric targeting of INCENP is not only important for prometaphase congression, which would be expected to involve centromere activity, but also for successful cytokinesis, which has no direct functional link with centromeres.

In metaphase cells, centromeres occupy the region of the spindle with the highest concentration of antiparallel microtubules. This is the region where the stem body assembles, and the centromeres may present INCENP to the spindle in a location or conformation that is required for stem body assembly. The failure of INCENP83–839 to be specifically transported to this domain of the spindle by chromosomes may explain why this protein fails to target to the midzone and instead coats the entire spindle during mitosis (Fig. 5).

Are Chromosomes Necessary for Cytokinesis?

When microsurgery was used to remove all chromosomes from insect spermatocytes in meiosis I after the establishment of a bipolar spindle, the cells appeared to execute anaphase and cytokinesis normally (Zhang and Nicklas, 1996). Although this experiment appeared to argue against a role for chromosomes in the later stages of mitosis, the results are subject to two caveats. First, cytokinesis in spermatocytes does not normally go to completion, at least in mammals. Developing spermatocytes and spermatids remain connected by intercellular bridges 2 or 3 μm in diameter until the time the sperm are shed (Dym and Fawcett, 1971; Dym, 1988). Thus, the process may differ in important respects from that in somatic cells with complete cytokinesis. Second, the experiments did not include an immunohistochemical analysis for either INCENP or TD-60. If these proteins are, in fact, present in meiotic cells, one or both may detach from the chromosomes either during microsurgery or in vivo at the time of bipolar spindle assembly, thus permitting them to perform their cytoskeletal functions normally even after the removal of the chromosomes. Third, it is possible that even if present, INCENP and TD-60 act preferentially at meiosis II, the division at which sister centromeres disjoin (and which was not examined in the Zhang and Nicklas [1996] study). It will be important in future experiments to determine the fate of INCENP in grasshopper spermatocytes after removal of the chromosomes.

Two other recent studies have also indicated that furrowing in mitotic cells may not require the local presence of a spindle midzone with segregating chromosomes. In one study (Eckley et al., 1997), when adjacent mitotic human osteosarcoma cells were fused manually by needle puncture, they formed V-shaped spindles upon which chromosomes segregated apparently normally. In 4 out of 19 of those cells (21%) an ectopic furrow formed above the open end of the V, where the two poles were connected by a microtubule array that lacked segregating chromosomes. In the second study, PtK1 cells were electrofused, and it was noted that if the spindles were ≥20 μm apart in the heterokaryons, they stayed separate throughout mitosis (Rieder et al., 1997). In this case furrows formed between the adjacent spindles in 8 out of 30 cases (26%), even though this region of the cell lacked segregating chromosomes. In both cases, immunostaining of these cells for INCENP has revealed the presence of the protein in both the normal and ectopic furrows (Eckley et al., 1997) (Savoian, M., and C. Rieder, unpublished data), thereby revealing that even though the protein is localized...
to the chromosomes through metaphase, it can relocate to distant parts of the cell cortex as cells establish the cleavage furrow in ana/telophase. The mechanism for this INCENP relocalization is not presently known.

The reversal of cytokinesis after the initiation of furrowing caused by expression of INCENPΔ405 in mammalian cells resembles the phenotype seen in micromanipulation experiments using sand dollar eggs where the nucleus was separated from the asters (Rappaport, 1991). Those experiments led Rappaport to conclude that although asters alone are able to trigger the formation of a cleavage furrow, a nuclear factor was necessary to enable the furrowing to proceed to completion. It is tempting to speculate that this nuclear factor might be either INCENP or an INCENP-interacting protein.

The present studies suggest that INCENP is involved in both early and late mitosis, with a dominant-negative INCENP mutant affecting both chromosome congression and cytokinesis. Although INCENP is a nuclear/chromosomal protein for most of the cell cycle, it is required for at least one cytoskeletal function in mitosis. Interestingly, the ability of INCENP to execute this cytoskeletal function requires previous localization of the protein to centromeres. These results are consistent with the hypothesis that one function of INCENP and other chromosomal passenger proteins may be to integrate the chromosomal and cytoskeletal events of mitosis.

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