Kinetochore Chemistry Is Sensitive to Tension and May Link Mitotic Forces to a Cell Cycle Checkpoint

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Abstract. Some cells have a quality control checkpoint that can detect a single misattached chromosome and delay the onset of anaphase, thus allowing time for error correction. The mechanical error in attachment must somehow be linked to the chemical regulation of cell cycle progression. The 3F3 antibody detects phosphorylated kinetochore proteins that might serve as the required link (Gorbsky, G. J., and W. A. Ricketts. 1993. *J. Cell Biol.* 122:1311–1321). We show by direct micromanipulation experiments that tension alters the phosphorylation of kinetochore proteins. Tension, whether from a micromanipulation needle or from normal mitotic forces, causes dephosphorylation of the kinetochore proteins recognized by 3F3. If tension is absent, either naturally or as a result of chromosome detachment by micromanipulation, the proteins are phosphorylated. Equally direct experiments identify tension as the checkpoint signal: tension from a microneedle on a misattached chromosome leads to anaphase (Li, X., and R. B. Nicklas. 1995. *Nature (Lond.).* 373:630–632), and we show here that the absence of tension caused by detaching chromosomes from the spindle delays anaphase indefinitely. Thus, the absence of tension is linked to both kinetochore phosphorylation and delayed anaphase onset. We propose that the kinetochore protein dephosphorylation caused by tension is the all clear signal to the checkpoint. The evidence is circumstantial but rich. In any event, tension alters kinetochore chemistry. Very likely, tension affects chemistry directly, by altering the conformation of a tension-sensitive protein, which leads directly to dephosphorylation.

The regulation of cell activities in response to mechanical force is a common cellular necessity. Responsive cells include endothelial cells lining the aorta (Rogers et al., 1985), hair cells in the ear (Hudspeth and Gillespie, 1994), and muscle cells adapting to exercise by synthesizing specific proteins (Goldspink et al., 1994). Nowhere is the connection between mechanics and regulation more essential than in mitosis. And in mitosis, as we show here, mechanical force leads directly to chemical change.

Accurate chromosome distribution in mitosis depends on the proper mechanical attachment of each chromosome to the spindle. A checkpoint in many cells monitors chromosome attachment and delays anaphase if an error is detected (Hartwell and Weinert, 1989; Murray, 1994). The delay provides time for error correction. A common error is the attachment of a chromosome to only one spindle pole rather than to both poles. Even a single such chromosome delays anaphase in cells as evolutionarily divergent as insect spermatocytes and vertebrate cells in culture (Callan and Jacobs, 1957; Zirkle, 1970; Rieder et al., 1994; Li and Nicklas, 1995). Similar sensitivity extends to budding yeast cells in which a single chromosome with defective kinetochores delays the exit from mitosis, very likely because of improper spindle attachment (Neff and Burke, 1992; Spencer and Hieter, 1992). The organisms studied are few but diverse, so the ability to detect and respond to a single misguided chromosome evidently is a widespread capability of eukaryotic cells. It is not universal, however, since sea urchin oocytes proceed to anaphase without delay even when numerous unattached chromosomes are present (Sluder et al., 1994). Our focus is on the sensitive cells, those that somehow detect a single misattached chromosome.

The problem for the cell and for us is to connect mechanical errors with chemical change. The difference between proper and improper chromosome attachment is purely mechanical, while the checkpoint is part of a chemical engine that drives the cell cycle. We seek the connection, the process by which a mechanical situation is transduced into a signal, presumably a chemical signal, that is detected by the checkpoint.

The first question is what is detected. There are several possibilities, not only the faulty attachment itself but various consequences of faulty attachment. Chromosomes attached to only one spindle pole lie in a distinctive position

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near that pole and unlike chromosomes attached to both poles, they are not under tension from mitotic forces toward opposite poles. McIntosh (1991) and later Rieder et al. (1994) suggested that it is the absence of tension that signals the checkpoint. The tension proposal was tested directly by micromanipulation of praying mantid spermatocytes (Li and Nicklas, 1995). The three sex chromosomes in mantid spermatocytes sometimes fail to remain connected, resulting in cells with an unpaired chromosome connected to only one spindle pole. Such cells are delayed in entering anaphase by several hours and eventually degenerate, which prevents the production of sperm with aberrant sex chromosome complements (Callan and Jacobs, 1957; Li and Nicklas, 1995). Pulling on the unpaired sex chromosome with a micromanipulation needle puts the chromosome under tension. Anaphase follows, several hours in advance of anaphase in sister cells with an unpaired sex chromosome that was not manipulated (Li and Nicklas, 1995). Thus, tension makes the misbegotten pass for normal: the checkpoint no longer detects an inhibitory signal and anaphase follows—even though improper chromosome combinations often result.

The second question is how tension signals the checkpoint. Some evidence points to the kinetochore or the centromere around it as the source of the signal: anaphase is delayed by mutant kinetochores in yeast (Spencer and Hieter, 1992) and by the injection of anti-centromere antibodies (Bernat et al., 1990; Yen et al., 1991; Tomkiel et al., 1994). A kinetochore component with just the required properties for a link between mechanics and the cell cycle has recently been discovered (Gorbsky and Ricketts, 1993). An antibody, 3F3, recognizes certain kinetochore proteins only when they are phosphorylated. The proteins are phosphorylated and detectable before the attachment of chromosomes to the spindle and they become dephosphorylated as chromosomes attach properly and move to the spindle equator (Gorbsky and Ricketts, 1993). Most significantly for checkpoint control, the kinetochore proteins of misattached chromosomes remain phosphorylated and stain brightly after immunostaining. The bright-staining, phosphorylated state of misattached kinetochores might be the signal to the checkpoint to delay division. Consistent with that possibility, microinjection of cells with the 3F3 antibody inhibits the dephosphorylation of kinetochore proteins and delays the onset of anaphase (Campbell and Gorbsky, 1995).

Our approach to the significance of the protein phosphorylation identified by 3F3 is to determine its relationship to mitotic mechanics. We combined micromanipulation and 3F3 immunostaining to study directly how attachment and its consequences affect kinetochore protein phosphorylation. Grasshopper spermatocytes, the material of choice for micromanipulation, show the same attachment-sensitive phosphorylation/dephosphorylation observed in mammalian cells (Gorbsky and Ricketts, 1993). Pulling on chromosomes with a micromanipulation needle provides direct evidence that it is tension that leads to dephosphorylation of the protein(s) recognized by 3F3 rather than attachment itself. On the basis of numerous correlations, we propose that tension-sensitive phosphorylation is the signal to the checkpoint. That remains to be proven directly, but our evidence that mechanical force can specifically alter kinetochore chemistry is unequivocal.

Materials and Methods

Materials, Micromanipulation, and Living Cell Observations

Spermatocytes from laboratory colonies of the grasshopper *Melanoplus sanguinipes* (Fabricius) were cultured at a temperature of 23–25°C under oil as previously described (Nicklas and Ward, 1994 and references therein). The spermatocytes were viewed by phase contrast microscopy and micromanipulated by standard procedures (Nicklas and Ward, 1994 and references therein). Chromosome behavior was recorded on an optical disk recorder (model 2021; Panasonic Video Systems, Secaucus, NJ).

Immunofluorescence Procedures

Immunostaining with the monoclonal 3F3/2 antibody (Cyert et al., 1988) was carried out largely as described by Gorbsky and Ricketts (1993) but some modifications were necessary. Cultured spermatocytes do not adhere to the coverslip on which they lie, and hence they cannot be rinsed and lysed before fixation as in the Gorbsky and Ricketts procedure. Instead, cells were lysed and fixed simultaneously by microinjecting lysis/fixative buffer near them ("microfixation"; Nicklas et al., 1979); the presence of fixative makes the lysed cell's contents stick to the coverslip. The lysis/fixation buffer contained 1.5 × PHEM[1], 2% CHAPS detergent (Sigma Chem. Co., St. Louis, MO), 0.15% glutaraldehyde (Polysciences Inc., Warrington, PA), 2% formaldehyde (freshly prepared from paraformaldehyde; Fisher, Raleigh, NC) and 10 μM microcystin LR (GIBCO BRL, Gaithersburg, MD). When microinjected near the cells, the components of this solution are diluted by the culture fluid bathing the cells, hence the higher than usual concentrations. After 10 min, the oil covering the cells was flushed away with PHEM plus 1% formaldehyde and the coverslip was immersed in that solution for 5 min ("macrofixation"; Nicklas et al., 1979). The coverslips were rinsed three times in MBST and stored in the refrigerator in MBST for up to 3 h; otherwise, all steps in the procedure were carried out at room temperature. Next the cells were treated with 1% BSA (bovine serum albumin, Sigma) in MBS for 30 min and then were labeled with an ascites preparation of 3F3/2 antibody at a dilution of 1:2,000 in MBS with 1% BSA for 45 min. Following a rinse in MBST and 20 min in fresh MBST, the preparations were exposed for 45 min to Cy3-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) at a dilution of 1:50 in MBS with 1% BSA. Following a rinse in MBST and 20 min in fresh MBST, the preparations were rinsed in distilled water and stained for 5 min in 0.6 μg/ml DAPI (Sigma) in distilled water. After a final rinse in distilled water, the coverslips were mounted on slides with Vectashield (Vector Laboratories, Burlingame, CA) supplemented with 10 mM $CaCl_2$ (for better preservation of chromatin; Gorbsky and Ricketts, 1993). The edges of the coverslips were sealed with nail polish.

Some cell cultures were fixed, rinsed, and then treated with 100 activity units per ml of protein phosphatase 1 in PHEM for 30 min at 37°C. As a control, additional cultures were treated identically except that 100 nM of the phosphatase inhibitor microcystin was present along with the phosphatase. The enzyme was provided by Dr. S. Shenolikar (Department of Pharmacology, Duke University).

The labeled cells were examined on an epifluorescence microscope (Axioplan; Carl Zeiss, Inc., Thornwood, NY) equipped with a Zeiss 100×/1.3 numerical aperture ICS Plan-Neofluar phase contrast objective and a cooled CCD video camera (model C4880; Hamamatsu Photonic Systems Corp., Bridgewater, NJ). Digital images were acquired with the Metamorph system (Universal Imaging Corp., West Chester, PA). In a representative sample of cells, the brightness of the kinetochore fluorescence was measured. Measurements were made in the raw, unprocessed images using Metamorph software. An area encompassing a kinetochore was defined and the total, integrated brightness was measured (i.e., the brightness values of individual pixels were summed for all pixels in the area). The integrated brightness of the kinetochore was corrected for background fluorescence by subtracting the brightness of a nearby area of the same size but lacking a kinetochore. The linearity of the relationship between measured pixel brightness and actual object brightness was verified by measuring the brightness of calibrated neutral density filters ranging from 97.7 to 2.3% transmission. The measured values differed by no more than 3% from the calibration value. The measurements were made on images at a single focal level with the 1.3 numerical aperture objective. The

1. *Abbreviations used in this paper:* PHEM, 60 mM Pipes, 25 mM Hepes at pH 6.95, 10 mM EGTA, and 4 mM $MgCl_2$; MBS, 10 mM Mops at pH 7.4 and 150 mM NaCl; MBST, MBS with 0.05% Tween 20.

focal depth of this objective is great enough to include the fluorescence from the full depth of a kinetochore; measurements with an objective with a numerical aperture of 0.9 (and hence substantially greater depth of focus) gave no higher values for kinetochore fluorescence than did the 1.3 aperture objective. Fluorescence and phase contrast images of the cells were stored as digital files and processed by computer, using commercial software (Photoshop; Adobe Systems Inc., Mountain View, CA and PowerPoint; by Microsoft Corp., Bellevue, WA). The contrast was adjusted and pairs of images were produced, one showing the fluorescence alone and the other a combined phase contrast/fluorescence view in which separate phase contrast and fluorescence images were superimposed. Often, the images are montages of two focal levels, so that several kinetochores can be compared in a single view. False color was added to the fluorescence images. Prints were made with a dye sublimation printer (Duke University Audio-Visual Services).

Results

Grasshopper Spermatocytes Have a Sensitive Checkpoint

Spermatocytes in the first meiotic division occur in cysts of 64 cells that progress synchronously through the cell cycle. In culture, the synchrony is imperfect but still makes detecting the effect of misattached chromosomes easy. We micromanipulated one cell in a group of cells in late prometaphase or metaphase and used the rest of the cells as controls (the number of control cells varied from 3 to 10, with an average of 7). In the manipulated cell, a chromosome was detached from the spindle with a microneedle. Such a detached chromosome lacks kinetochore microtubules and hence is completely disconnected from the spindle (Nicklas and Kubai, 1985). Ordinarily, a detached chromosome soon reattaches to the spindle, and if left unperturbed, comes back under tension from mitotic forces toward opposite poles in a few minutes (Nicklas and Ward, 1994). We repeatedly redetached the chromosome before tension was reestablished but made no attempt to keep the chromosome constantly free of spindle connections. In consequence, the manipulated chromosome was attached by one or both kinetochores ~80–90% of the time, but was not under tension (the absence of tension was obvious because the chromosome was not stretched). We describe such chromosomes as "relaxed."

If the cell was very close to anaphase when the experiment began, it soon divided despite the presence of a relaxed chromosome. We happened across three such cells. They entered anaphase 7.3–10 min (average 8.6 min) after a chromosome was detached. Detachment of a chromosome at any earlier time invariably delayed the onset of anaphase. In 11 experiments, a chromosome was kept relaxed by repeated detachments for at least 30 min after the last unmanipulated control cell divided; the average total time without tension was over two hours (123 min, range, 62–225 min). The delay in anaphase onset compared to controls averaged more than two hours (average 139 min, range 23–229 min). The difference in when anaphase began in cells with and without a relaxed chromosome is highly significant statistically (two-tailed *t*-test, paired values; the hypothesis of equal means is rejected with $P = 3 \times 10^{-5}$). The most stringent test is to use the very last control cell to enter anaphase as the standard for comparison. The average time between anaphase onset in the last control cell and in the cell with a relaxed chromosome was 102 min (range 13–211 min). Again, the difference is highly significant statistically (two-tailed *t*-test, paired values; $P = 4 \times 10^{-4}$). The maximum delay caused by a relaxed chromosome is over 3.5 h (211 min) in the experiments conducted to date.

Anaphase in cells with a relaxed chromosome occurred quite some time after the chromosome was released from the manipulation needle for the last time, an average of 53 min (range 11–112 min). Even after the chromosome reattached to the spindle and was again under tension from mitotic forces, an average of 43 min (range 5–89 min) elapsed before the onset of anaphase.

General Features of Antibody 3F3 Immunostaining in Spermatocytes

Kinetochore Immunostaining Is Strong in Prophase but Weakens After Chromosomes Attach to the Spindle and Are under Tension. As in mammalian PtK cells (Gorbsky and Ricketts, 1993), immunostaining with the 3F3 antibody lights up the kinetochores and poles of dividing spermatocytes. All kinetochores fluoresce brightly in late prophase (Fig. 1). Kinetochore fluorescence dims as chromosomes attach to the spindle, come under tension from mitotic forces, and move to the metaphase plate (Fig. 2). Unlike PtK cells (Gorbsky and Ricketts, 1993), however, some kinetochore fluorescence remains after chromosome attachment and movement (Fig. 2). Often, the axis between the chromatids is also fluorescent (Fig. 2). During anaphase, kinetochore 3F3 staining drops to a very low level and pole staining becomes greatly reduced (not illustrated).

Antibody 3F3 Recognizes Phosphoproteins in the Kinetochore and Elsewhere in Grasshopper Spermatocytes. Gorbsky and Ricketts (1993) discovered the conditions necessary for immunostaining kinetochores in mammalian cells with the 3F3 antibody; the same conditions apply to grasshopper spermatocytes: (*a*) the cells must be extracted before or during fixation to remove soluble antigen that otherwise obscures kinetochore staining, and (*b*) the protein phosphatase inhibitor microcystin must be present in the extraction medium; evidently it inhibits an endogenous phosphatase that otherwise dephosphorylates some of the proteins recognized by the 3F3 antibody. In spermatocytes, unlike mammalian cells (Gorbsky and Ricketts, 1993), some staining of kinetochores and centrosomes is seen if microcystin is omitted (not shown). Both unattached and attached kinetochores are dimly immunofluorescent: without microcystin the brighter fluorescence of unattached kinetochores (as in Fig. 1) is missing. Therefore all kinetochores appear the same regardless of stage and regardless of their functional state (i.e., whether or not they are attached to the spindle and whether or not are they are moving, etc.). We call this residual fluorescence "ground-level staining."

The specificity of antibody 3F3 for phosphoproteins can be tested by treating cell preparations with protein phosphatase 1 before immunostaining (Gorbsky and Ricketts, 1993). Protein phosphatase 1 abolishes all staining in grasshopper spermatocytes (Fig. 3, *a* and *b*). Additional evidence for specificity is found by using the phosphatase inhibitor microcystin along with the phosphatase; the normal, bright immunofluorescence is seen (Fig. 3, *c* and *d*). The cell in Fig. 3, *c* and *d* was fixed during chromosome attachment to the spindle and some kinetochores (*arrows*)

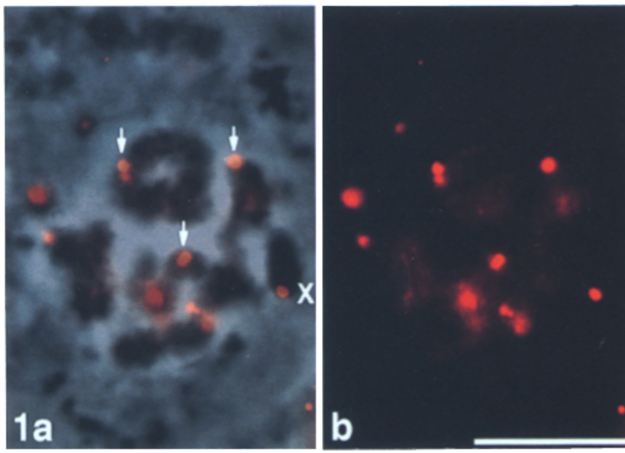

Figure 1. Kinetochores in prophase (diakinesis) are bright after 3F3 immunostaining. *(a)* 3F3 immunofluorescence image (*red*) superimposed on a phase contrast image to show the chromosomes. Some kinetochores of paired chromosomes (autosomes) are labeled (*arrows*) as is the unpaired X-chromosome's kinetochore (X). (*b*) Immunofluorescence alone. Bar, 10 μm.

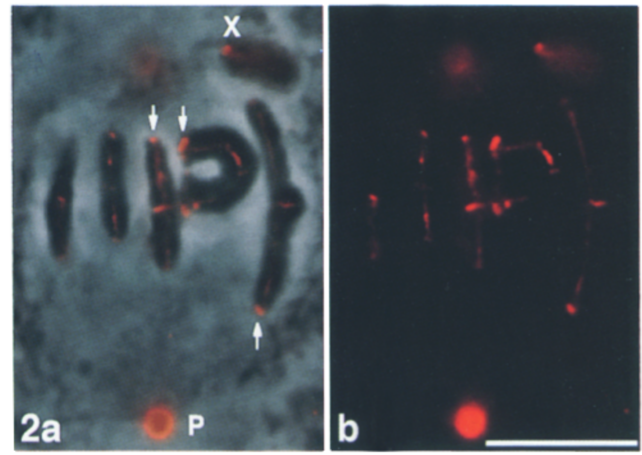

Figure 2. Kinetochores in metaphase are dim after 3F3 immunostaining. (*a*) Superimposed phase contrast and fluorescence (*red*) images. One pole (*p*) and some kinetochores (*arrows*, autosomes; *X*, X-chromosome) are labeled. (*b*) Immunofluorescence alone. Bar, 10 μm.

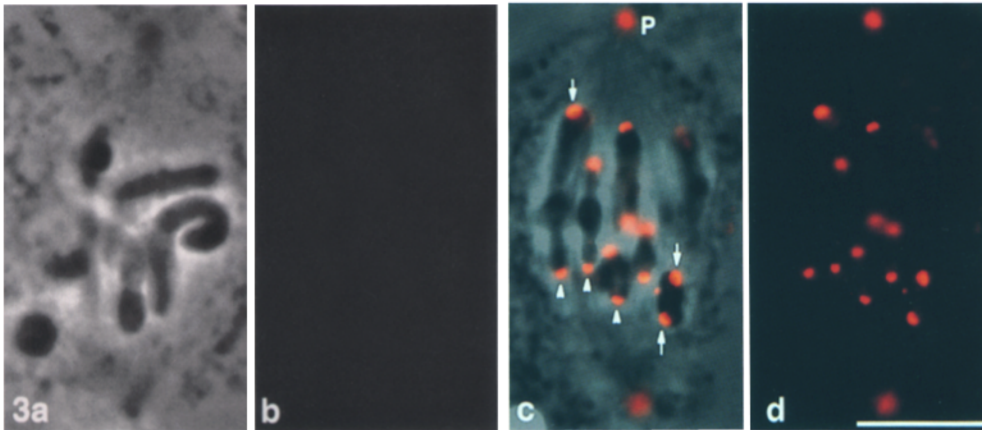

Figure 3. Digestion with protein phosphatase 1 abolishes 3F3 immunostaining unless the phosphatase inhibitor microcystin is present. Cells in early prometaphase. (*a* and *b*) 3F3 staining of a cell digested with protein phosphatase 1. (*a*) Phase contrast. (*b*) Immunofluorescence of the same cell. (*c* and *d*) 3F3 staining of a cell digested with protein phosphatase 1 in the presence of microcystin. (*c*) Superimposed phase contrast and fluorescence (*red*) images. One pole (*p*) and some kinetochores (*arrows*) are labeled. (*d*) Immunofluorescence alone. Bar, 10 μm.

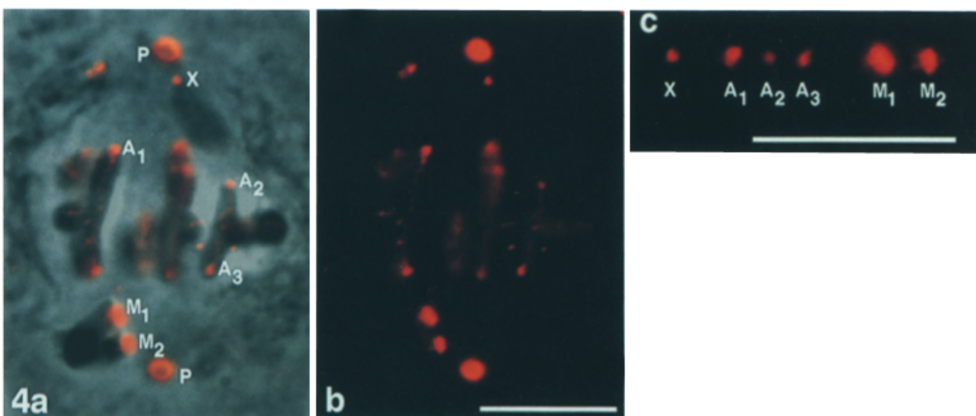

Figure 4. Kinetochores of an improperly attached chromosome have greater 3F3 immunofluorescence than those of properly attached chromosomes. (*a*) Superimposed phase contrast and fluorescence (*red*) images. M_1, M_2, kinetochores of a misattached chromosome (both kinetochores are attached to the same, nearby pole); A_1, A_2, A_3, kinetochores of properly attached chromosomes; *X*, X-chromosome's kinetochore; *P*, pole. (*b*) Immunofluorescence alone. (*c*) Composite at higher magnification showing the kinetochores labeled in *a*. Bar, 10 μm.

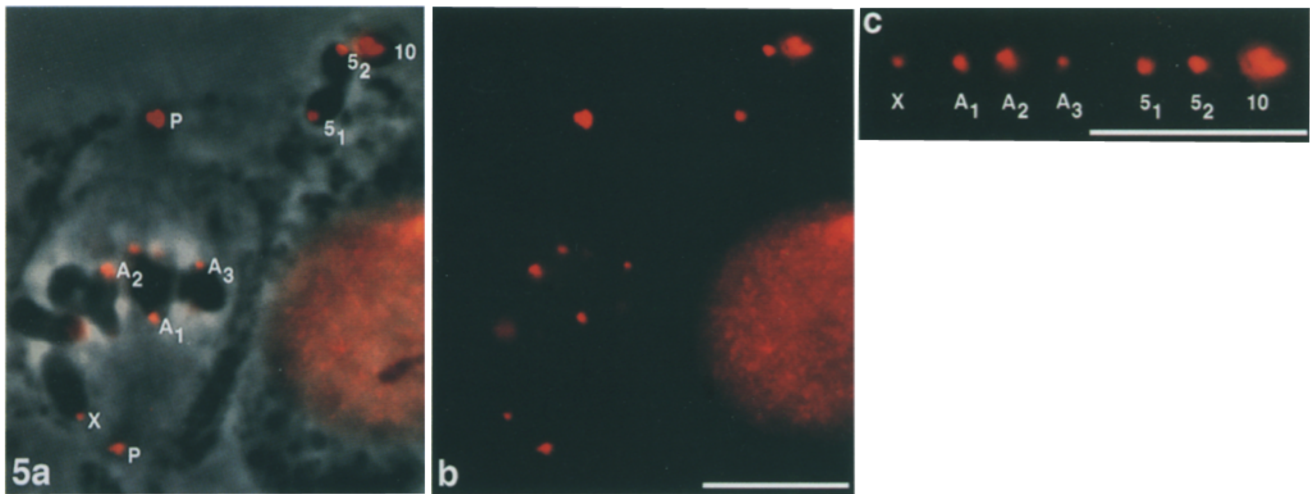

Figure 5. Strong 3F3 immunofluorescence reappears at kinetochores after chromosomes are detached from the spindle. (*a*) Superimposed phase contrast and fluorescence (*red*) images. Before fixation, two chromosomes were detached from the spindle by micromanipulation and kept from reattaching, one for 5 min (kinetochores labeled 5_1, 5_2) and one for 10 min (two closely appressed kinetochores labeled *10*). A_1, A_2, A_3, kinetochores of properly attached chromosomes; *X*, X-chromosome's kinetochore; *P*, poles. (*b*) Immunofluorescence alone. (*c*) Composite at higher magnification showing the kinetochores labeled in *a*. Bar, 10 μm.

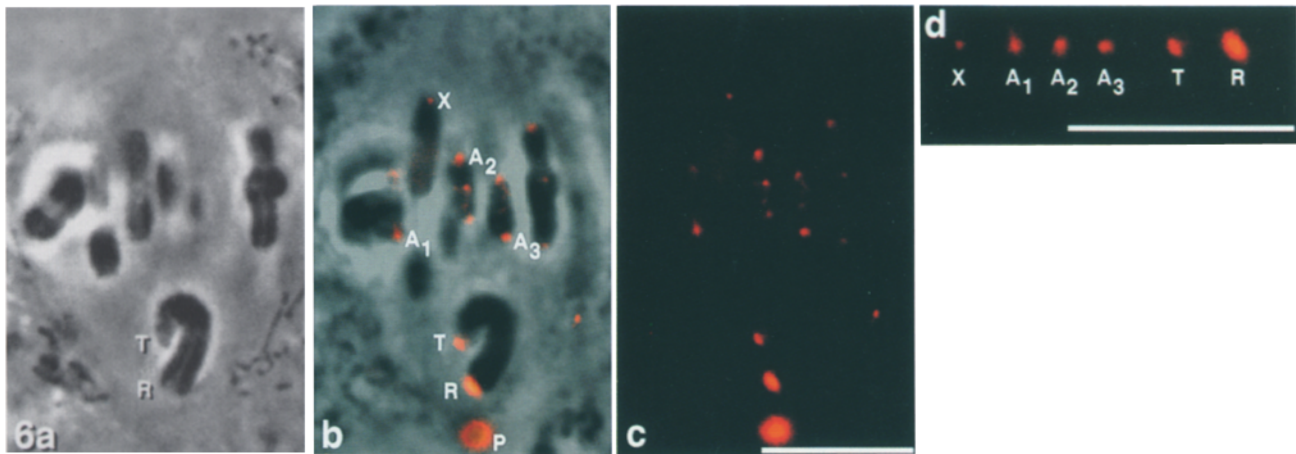

Figure 6. Tension applied by a micromanipulation needle causes dim 3F3 immunostaining. (*a*) Living cell with a misattached chromosome, as in Fig. 4. Both kinetochores of the misattached chromosome (*T* and *R*) were attached and anchored to the lower spindle pole, so that when a micromanipulation needle (too small to be seen) was inserted and moved upward, the chromosome was stretched (note tapered end at *T*). Tension was imposed on one kinetochore (*T*) but its partner remained relaxed (*R*). (*b*) Superimposed phase contrast and fluorescence (*red*) images. The relaxed kinetochore (*R*) stains brightly, but the kinetochore under tension (*T*) is smaller and dimmer, like those of properly attached, unmanipulated chromosomes (A_1, A_2, A_3). *X*, X-chromosome's kinetochore; *P*, pole. (*c*) Immunofluorescence alone. (*d*) Composite at higher magnification showing the kinetochores labeled in *a*. Bar, 10 μm.

fluoresce more brightly than others (*arrowheads*). In one chromosome (Figs. 3, *c* and *d, left*), one kinetochore is bright while its partner is dim. Notice that the fluorescent region of bright kinetochores is off-axis; that is, it is not centered at the end of the chromosome as is the fluorescence of dimly stained kinetochores (Fig. 3 *c*). This suggests that brightly stained kinetochores are not subjected to forces toward opposite poles that would bring the kinetochore and the axis of the chromosome into alignment.

Kinetochores of Misattached Chromosomes Have Greater 3F3 Immunofluorescence than Kinetochores of Properly Attached Chromosomes

Early in spindle formation, chromosomes with both kinetochores attached to the same spindle pole are common (reviewed by Nicklas, 1988). These incorrectly attached chromosomes and their kinetochores are not under tension from mitotic forces to opposite poles. The kinetochores of such chromosomes are conspicuous after 3F3 immunostaining Fig. 4, M_1, M_2). They are larger than the kinetochores of properly attached chromosomes (as seen by conventional, nonconfocal fluorescence microscopy) and often they are brighter (Fig. 4, A_1, A_2, A_3). The composite, Fig. 4 *c*, makes comparisons easier. We made quantitative comparisons by measuring the total brightness of kinetochores, i.e., the brightness of the fluorescence integrated over the whole volume of the kinetochore. The misattached kinetochores in Fig. 4 were about twice as bright as the kinetochores of the other, properly attached, chro-

mosomes: one kinetochore was 1.8 times brighter than the average for the kinetochores of chromosomes at the equator and the other was 2.3 times as bright. The total brightness of four misattached kinetochores was measured; on average, they were 2.1 times as bright as kinetochores of properly attached chromosomes in the same cell (Table I). Five additional examples confirm qualitatively the greater fluorescence, the higher 3F3-phosphoprotein content, of kinetochores in chromosomes that are attached to only one spindle pole and therefore are not under tension.

Kinetochore Protein Dephosphorylation Is Reversible—Bright Staining with 3F3 Returns After Chromosomes Are Detached from the Spindle

To test whether the kinetochore protein dephosphorylation associated with attachment to the spindle is reversible, we detached chromosomes from the spindle by micromanipulation. As already noted, detached chromosomes lack kinetochore microtubules (Nicklas and Kubai, 1985). Lacking any connection with the spindle, detached chromosomes are not under tension from mitotic forces. Two chromosomes were detached from the spindle in the cell shown in Fig. 5 and were kept detached (i.e., they were redetached following every spontaneous reattachment). One was kept detached for 5 min and the other for 10 min (Fig. 5 *a*, *5* and *10*), and then the cell was fixed for immunostaining. The 3F3 fluorescence of one kinetochore of the chromosome detached for 5 min (Fig. 5, *a* and *c*, 5_1) does not appear to differ much from that of the normally attached chromosomes on the spindle (Fig. 5, A_1–A_3), but measurements of total fluorescence reveal that it was 1.4 times as bright as the average brightness of kinetochores of normally attached chromosomes. The other kinetochores of the detached chromosomes are brighter and/or larger (Fig. 5, *a* and *c*, 5_2 and *10*). Their total fluorescence per kinetochore was 2.2–2.4 times that of normally attached chromosomes (the kinetochores of the small chromosome kept detached for 10 min were very close together [Fig. 5, *10*]; their combined fluorescence was measured and divided by two to give an average value for the fluorescence of each kinetochore).

Four kinetochores from chromosomes kept detached for 5 min were studied and the brightness of all four was measured; on average they were 1.4 times as bright as the kinetochores of properly attached chromosomes (Table I). All 19 kinetochores of chromosomes kept detached for 6–15 min stained brightly with 3F3. Six of these kinetochores were measured; on average they were 2.2 times brighter than the kinetochores of normally attached chromosomes (Table I). Thus, the dephosphorylation associated with attachment is reversible, but slowly: maximal rephosphorylation after detachment generally requires longer than 5 min but less than 10 min.

Table I. Fluorescence Measurements

Situation	Example	Under tension?	No. of measurements	Fluorescence per kinetochore*
Misattached	Fig. 4	No	4	2.1 ± 0.2
Detached				
5 min	Fig. 5, *5*	No	4	1.4 ± 0.5
10–13.5 min	Fig. 5, *10*	No	6	2.2 ± 0.5
Unequal tension				
Relaxed	Fig. 6, *R*	No	6	2.4 ± 0.5
Under tension	Fig. 6, *T*	Yes	6	1.1 ± 0.2
X-chromosome	Figs. 4–6	No	6	0.70 ± 0.1

*The total brightness (above background) of each kinetochore was measured and was standardized by dividing by the average total brightness of properly attached kinetochores in the same cell (e.g., the kinetochores of misattached chromosomes were 2.1 times as bright, on average, as properly attached chromosomes). The values are mean ± 95% confidence limits.

Tension from a Microneedle Causes Kinetochore Protein Dephosphorylation

We experimentally produced cells in which one chromosome had both kinetochores attached to the same pole. The chromosome was detached from the spindle and bent so that both of its kinetochores faced the same pole. It was kept completely relaxed (not under tension) for 10 min, to allow time for rephosphorylation of the kinetochore protein recognized by 3F3. During this time, both kinetochores spontaneously attached to the nearby pole (Ault and Nicklas, 1989). The result is an improperly attached chromosome that is just like those that occur naturally (Fig. 4), and tension from mitotic forces toward opposite poles is missing. In these experiments, however, we supplied the missing tension by micromanipulation. A microneedle was inserted into the chromosome and moved toward the equator, stretching the chromosome. To provide an internal control, the needle was positioned so that one kinetochore was placed under tension (Fig. 6 *a*, *T*) while its partner was not (Fig. 6 *a*, *R*). After 6 min, the cell was fixed and stained. The composite, Fig. 6 *d*, facilitates comparisons of kinetochore fluorescence. The fluorescence of the kinetochore that was under tension (Fig. 6, *b* and *d*, *T*) was similar to that of normally attached chromosomes (Fig. 6, *b* and *d*, *A*) while its nearby, relaxed partner had a much larger and brighter fluorescent area (Fig. 6, *b* and *d*, *R*). Measurements disclose that the kinetochore under tension was no brighter than the average for unmanipulated chromosomes (the ratio of fluorescence was 1.0) while the relaxed chromosome was 2.2 times as fluorescent. In all eight such experiments, tension invariably reduced the fluorescence of one kinetochore as compared to its relaxed partner. The total kinetochore fluorescence was measured in six experiments. On average, the kinetochore under tension was 1.1 times as bright as properly attached controls while the relaxed kinetochore was 2.4 times as bright (Table I).

The X-chromosome Is Exceptional: Its Kinetochore Proteins Are Dephosphorylated Even Though Tension Presumably Is Absent

The X-chromosome in grasshopper spermatocytes is unpaired, and consequently it is attached to only one pole. In the absence of a second kinetochore attached to the opposite pole, it would be expected that tension from mitotic forces is missing and hence the X-chromosome's kinetochore might be expected to be bright after 3F3 immunostaining. In fact, the X-chromosome's kinetochore is dim, somewhat dimmer even than the kinetochores of chromo-

somes at the metaphase plate and under tension (Figs. 2, 4, 5, and 6, *X*). A particularly revealing example is the X-chromosome in Fig. 4. Although it is presumably not under tension, as is true of the misattached chromosome at the opposite pole, the X-chromosome's kinetochore (*X*) is dim while those of the misattached chromosome (M_1, M_2) are bright. The fluorescence of six X-chromosome kinetochores was measured; the values ranged from 0.5 to 0.9 times the values for autosomal chromosomes under tension (average 0.70).

Fluorescence Measurements

The average brightness due to 3F3 immunostaining of a representative, small sample of kinetochores in various situations is given in Table I. Except for the X chromosome, the absence of tension is associated with phosphorylated kinetochores: uniformly they are about twice as bright as the kinetochores of properly attached chromosomes. This is true whether tension is absent naturally (*Misattached*) or because a chromosome had been detached from the spindle for longer than 5 min (*Detached, 10–13.5 min* and *Unequal tension, relaxed*). After detachment for only 5 min, the rephosphorylation of the protein recognized by 3F3, is just starting and the kinetochores are intermediate in brightness between properly attached and relaxed kinetochores. Tension applied with a micromanipulation needle is just as effective as mitotic forces in reducing kinetochore phosphorylation—the brightness is indistinguishable statistically from 1.0 (Table I, *Unequal tension, under tension*), which is the standardized brightness of properly attached kinetochores.

Discussion

The Mid-Mitosis Quality Control Checkpoint

Grasshopper spermatocytes now join the short list of cells with a verified checkpoint that is sensitive to a single misattached chromosome. Earlier reports on grasshopper spermatocytes were equivocal. At first, it was thought likely that a misattached chromosome delays the start of anaphase (Nicklas, 1967). This was later denied (Nicklas and Arana, 1992), however, because cells with misattached chromosomes were observed to enter anaphase. Those observations showed that an absolute block does not exist, but a delay was not ruled out. Our present experiments were designed to test for any delay in the start of anaphase due to the presence of a chromosome we describe as relaxed; a chromosome was detached from the spindle by micromanipulation and then manipulated so that for most of the time it was attached to the spindle but was not under tension. A delay proved easy to detect. The presence of a single relaxed chromosome has so potent an effect on anaphase onset that the delay overwhelms some variability in when the normal control cells enter anaphase. The start of anaphase was delayed by as much as three and a half hours. This is not the maximum possible delay the checkpoint can induce, but rather it is just the duration of the longest experiment we have so far performed.

The existence of a checkpoint in grasshopper spermatocytes is surprising. All the cells have a chromosome, the X-chromosome, which is attached to only one pole and should be detected by the checkpoint, delaying division indefinitely. As discussed below, the X-chromosome may be invisible to the checkpoint.

Tension Affects Kinetochore Chemistry

The antibody 3F3 recognizes a certain set of phosphoproteins in both a mammalian cell line (Gorbsky and Ricketts, 1993) and in grasshopper spermatocytes. Treatment with protein phosphatase 1 abolishes 3F3 immunostaining, but staining is normal if the phosphatase inhibitor microcystin is present along with the phosphatase. Several cellular components contain phosphoproteins recognized by 3F3 and hence the cells must be extracted with detergent to reveal state-specific kinetochore staining. The specificity of the staining testifies that it is not an artifact due to protein relocation during extraction. For example, differential staining of adjacent kinetochores is correlated with the presence or absence of tension before the cell was lysed (Fig. 6); it is hard to imagine that this correlation is a product of protein relocation during extraction after lysis.

As in a mammalian cell line (Gorbsky and Ricketts, 1993), kinetochore immunostaining with the antibody 3F3 in grasshopper spermatocytes is sensitive to the functional status of the kinetochore. The overall pattern is that bright kinetochore staining appears in prophase, diminishes as chromosomes become attached to the spindle and come under tension to opposite poles, and disappears during anaphase. Chromosomes that have attached to the spindle, but improperly, so that they are not under tension, have brightly staining kinetochores (Fig. 4). In sum, certain kinetochore proteins recognized by 3F3 are phosphorylated before chromosomes attach to the spindle and become dephosphorylated after proper attachment and movement.

Such observations leave in doubt the cause of state-specific phosphorylation and dephosphorylation. The decrease in phosphorylation detected by 3F3 as chromosomes attach to the spindle could reflect some feature of attachment itself or some consequence of attachment such as chromosome movement, kinetochore position on the spindle, or the tension due to forces toward opposite poles that follows proper attachment. Chromosome micromanipulation provides direct evidence that the dephosphorylation is due to tension. Chromosomes with both kinetochores attached to the same spindle pole were stretched with a microneedle so that one kinetochore was under tension while the other was not (Fig. 6). The two kinetochores are otherwise very similar. They are near one another, both are attached to the spindle, and neither is moving much. The difference is tension; the kinetochore under tension from a microneedle is dim, while its relaxed partner is bright (Fig. 6, *b* and *d*), twice as bright, according to measurements of total fluorescence. In addition, micromanipulation discloses that the kinetochore dephosphorylation caused by tension is reversible. After chromosomes are detached from the spindle, their kinetochore proteins slowly become rephosphorylated, and between 5 and 10 min after detachment they again fluoresce brightly after 3F3 immunostaining (Fig. 5; Fig. 6, *b* and *d*, *R*).

A possible alternative to a direct effect of tension on protein phosphorylation can probably be dismissed. The

protein(s) recognized by 3F3 might be part of the kinetochore's corona, which has been postulated to stretch out toward the pole when tension is present (Rieder and Alexander, 1990). If that were true, the proteins would be spread out in space and thus diluted, perhaps diluted enough to be undetectable by the antibody. In seeming accord with this, the fluorescent region of relaxed kinetochores stained by 3F3 is larger than that of kinetochores under tension. This size difference is at least partly an artifact of fluorescence microscopy, which inflates the apparent size of bright objects. Direct evidence against an extensible corona is available for kinetochores in a mammalian cell; electron microscopy shows that the 3F3 antigen is concentrated in the middle layer of the kinetochore, not the corona (Campbell and Gorbsky, 1995). Moreover, kinetochore staining often is sufficiently intense in both mammalian cells (Gorbsky and Ricketts, 1993) and grasshopper spermatocytes that a stretched-out corona should be easily seen, at least in the early stages of extension. Such extended fluorescent kinetochores have not been observed. We conclude that the changes associated with tension and its absence that are recognized by 3F3 all but certainly reflect genuine chemical changes in the kinetochore, i.e., the presence or absence of phosphorylated protein(s), rather than changes due to distribution in space.

There are two exceptions to the relationship between phosphorylation and tension. The first is the dim fluorescence of the unpaired X-chromosome, an understandable and illuminating exception, as discussed below. The second exception is the existence of chromosomes with one phosphorylated and one dephosphorylated kinetochore (Gorbsky and Ricketts, 1993; our Fig. 3, *c* and *d*). Differences between kinetochores in the same chromosome cannot be explained by tension toward opposite poles at the time when the cell was fixed, because if one kinetochore is under tension, so is its partner. The chromosomes with one phosphorylated and one dephosphorylated kinetochore occur early in the process of chromosome attachment to the spindle, when attachments are labile (Nicklas and Ward, 1994) and hence tension may come and go. The variation in tension may make kinetochore phosphorylation states unstable, but exactly how this would lead to differences between the two kinetochores remains a mystery. Tension evidently is not the only factor that regulates the phosphorylation of kinetochore proteins early in the attachment process.

Otherwise, the relationship between tension and phosphorylation is straightforward. Tension is absent when chromosomes are not attached to the spindle and as expected their kinetochore proteins are phosphorylated and stain brightly with 3F3. This holds for kinetochores of prophase chromosomes, of chromosomes that are tardy in attaching to the spindle, and of chromosomes that have been detached from the spindle by micromanipulation. Conversely, the kinetochores of chromosomes under tension, whether from normal mitotic forces (Fig. 2) or from a micromanipulation needle (Fig. 6) have dephosphorylated kinetochores as shown by dim 3F3 staining.

Protein Conformation and Tension: The Mechanism of Protein Phosphorylation Changes

A key to the mechanism of tension-sensitive changes in kinetochore phosphorylation is that the changes are determined locally, not globally. A striking example is the difference between adjacent kinetochores in Fig. 6. Each kinetochore responds to its own state, not to a global cellular signal that would affect all kinetochores.

One possibility is that tension does not actually cause dephosphorylation. Instead, tension might cause the phosphoprotein to become inaccessible to the antibody or to be lost from the kinetochore (Gorbsky and Ricketts, 1993). The strict requirement for a phosphatase inhibitor in preparing cells for 3F3 immunochemistry argues for true dephosphorylation. Confirming Gorbsky and Ricketts (1993), we find that the presence of the phosphatase inhibitor microcystin after cell lysis is necessary and sufficient to preserve the bright fluorescence of improperly attached kinetochores. In the absence of the inhibitor, all kinetochores have the same, relatively dim fluorescence. Evidently the inhibitor is necessary to prevent the selective dephosphorylation of the tension-sensitive phosphoprotein, presumably by a cellular phosphatase that is activated upon lysis of the cells. Thus, after lysis, the cell has the components necessary to specifically dephosphorylate the tension-sensitive kinetochore protein. We conclude that those components are very likely used to dephosphorylate kinetochores as they come under tension in living cells.

If dephosphorylation is the mechanism, it could be either indirectly or directly related to tension. An interesting possibility for indirect action is that tension might stabilize kinetochore microtubules, leading to an increase in their number when tension is present. The vacant microtubule binding sites when tension is absent might activate phosphorylation of the protein detected by 3F3. This possibility is ruled out in grasshopper spermatocytes by experiments in which the tension on chromosomes in living cells was correlated with the number of kinetochore microtubules as seen by electron microscopy. The number was unchanged in chromosomes fixed while under greater tension (Nicklas, R. B. and D. F. Kubai, unpublished observation).

Alternatively, tension might affect kinetochore phosphorylation directly. In that case, the evident mechanism is a change in conformation in a sensitive peptide produced simply by pulling on it. For instance, the 3F3 protein might be sensitive to tension. Pulling on it might alter its conformation, exposing a phosphorylation site to the action of an unlocalized phosphatase. Alternatively, the phosphatase might be sensitive to tension and be activated when it is stretched. The phosphatase would be located in the kinetochore (or in any part of the chromosome between the partner kinetochores that is stretched when tension is present). In fact, protein phosphatase 1 is specifically relocated from the cytoplasm to the chromosomes during mitosis in a mammalian cell line (Fernandez et al., 1992). Notice, however, that a specific location in the kinetochore or centromere would be more impressive for an agent that affects one kinetochore's chemistry without affecting nearby kinetochores.

We conclude that while alternatives are not ruled out, tension very likely affects kinetochore phosphorylation directly, by producing a conformational change in a tension-sensitive protein that leads to the dephosphorylation of the protein detected by 3F3.

Tension-sensitive Kinetochore Phosphorylation May Control a Cell Cycle Checkpoint

There are several alternatives for the biological role of the kinetochore phosphorylation states revealed by 3F3. The possibilities include regulation of kinetochore-microtubule interactions, regulation of kinetochore motors, or regulation of the cell cycle checkpoint (Gorbsky and Ricketts, 1993). Our results make checkpoint regulation a most attractive possibility. Phosphorylation status and the checkpoint are correlated as follows:

Misattached Chromosomes Have Phosphorylated Kinetochores and They Affect the Checkpoint. This is not a very revealing correlation, since misattached chromosomes have other distinctive properties that might be detected by the checkpoint, such as attachment to only one pole and position on the spindle.

A More Significant Correlation Is That Both Phosphorylation and the Checkpoint Respond to Tension and Share the Same Unit of Response: One or Two Kinetochores. (*a*) In the absence of tension, kinetochores are phosphorylated, and the checkpoint inhibits the onset of anaphase. The direct evidence for this is the phosphorylation of the kinetochore not under tension in Fig. 6 and the inhibition of anaphase onset in mantids when a single kinetochore is attached but is not under tension (Li and Nicklas, 1995). (*b*) Tension causes the dephosphorylation of a single kinetochore (Fig. 6), and the application of tension to a chromosome having but one kinetochore lifts the inhibition of anaphase onset in mantids (Li and Nicklas, 1995). (*c*) Detaching a chromosome from the spindle reverses its effect on the checkpoint, transforming a chromosome that was not inhibiting the onset of anaphase to one that inhibits anaphase onset indefinitely. Detaching a chromosome also leads to rephosphorylation of the 3F3 kinetochore protein, making a detached chromosome resemble misattached chromosomes that inhibit anaphase onset. So far, the smallest tested unit for this effect is the two partner kinetochores of one bivalent.

The X-chromosome of Grasshopper Spermatocytes is Exceptional. Like other organisms that have no Y-chromosome to pair with the X, the X-chromosome in grasshopper spermatocytes is attached to only one spindle pole and evidently is not under tension. Yet the X-chromosome does not delay anaphase. Contrast this with the situation in praying mantids, close relatives of the grasshoppers. In mantids with three sex chromosomes, spermatocytes with an X-chromosome attached to only one pole occur but are abnormal; the X is detected by the checkpoint, anaphase is delayed, and the eventual outcome is the degeneration of the afflicted cells without forming sperm. If the checkpoint worked that way in grasshoppers and other organisms with no Y-chromosome, no sperm would be formed. An obvious solution would be to suppress the checkpoint in spermatocytes. However, as we have shown, grasshopper spermatocytes have a checkpoint sensitive to a single misattached chromosome. An elegant alternative solution to the problem would be to retain the checkpoint but to silence the X-chromosome so that it, uniquely, sends no signal about whether or not it is under tension. If so, any proposed chemical signal to the checkpoint should be absent in the X-chromosome of grasshoppers, but it should be present in the X-chromosome of mantids when they are unpaired. The 3F3 phosphorylation patterns match these expectations exactly. In grasshoppers, the X-chromosome's kinetochore proteins are dephosphorylated and stain dimly, just like the kinetochores of properly attached chromosomes and of chromosomes under tension from a micromanipulation needle (Figs. 2 and 4–6). In mantids, however, the kinetochores of X-chromosomes that are attached to only one pole are phosphorylated and stain brightly, just as do the kinetochores of other misattached chromosomes; hence, they express the putative signal to the checkpoint (Li, X., and R. B. Nicklas, personal communication).

It might be thought that the X-chromosome in grasshopper spermatocytes actually is under tension, e.g., from ejection forces acting from the nearby pole toward the equator. Such forces certainly exist in some cells (Rieder et al., 1986). If they exist in spermatocytes, however, they are not sufficiently strong to perceptibly stretch the X-chromosome or even to align it with the pole-to-pole axis (e.g., Fig. 2). Moreover, any such force should also affect chromosomes like the misattached one in Fig. 4. Yet its kinetochores (M_1, M_2) are bright while those of the X-chromosome in the same cell (*X*) are dim.

In sum, kinetochores that affect the checkpoint invariably have phosphorylated 3F3 proteins while kinetochores that do not affect it are dephosphorylated.

None of the Other Identified Features of Misattached Chromosomes Is by Itself Sufficient to Trigger the Checkpoint or to Affect the Phosphorylation of the Protein(s) Recognized by 3F3. For instance, mere attachment of chromosomes to the spindle is not sufficient to signal the checkpoint to proceed to anaphase in the absence of tension (Li and Nicklas, 1995), nor is it sufficient to dephosphorylate the kinetochore (Figs. 4 and 6).

Two apparent contradictions to the correlation of tension-sensitive phosphorylation with the checkpoint signal merit discussion. In some cells the onset of anaphase is delayed in the presence of very low concentrations of microtubule inhibitors despite the attachment of all chromosomes and their proper movement to the spindle equator (Jordan et al., 1993; Wendell et al., 1993). Electron microscopic examination of cells treated with one inhibitor, vincristine, disclosed that the number of kinetochore microtubules is reduced (Wendell et al., 1993). A plausible explanation for delayed anaphase is that the reduced number of kinetochore microtubules leads to lower than normal mitotic forces and thus to tension that is too low to signal the checkpoint that all is in order (Rieder et al., 1994).

The second possible contradiction concerns the character of mitotic forces. The elegant analysis of Skibbens et al. (1993) shows that the oscillatory movements of chromosomes seen in some cells are associated with fluctuations in mitotic forces. Sometimes tension lapses and the kinetochores are under compression rather than tension. Now if kinetochore phosphorylation changed quickly, a lapse in tension would soon result in rephosphorylation, which would be followed by dephosphorylation when the kinetochore came under tension again. The result would be confusing signals to the checkpoint, a signal to delay anaphase being followed by a signal to proceed and so on. Such confusion could be avoided by appropriate kinetics for re-

phosphorylation, so that rephosphorylation is slow compared to the duration of lapses in tension. Just this is seen: our observations show that tension must be absent for 5–10 min before rephosphorylation occurs. In contrast, tension is absent in the kinetochores of oscillating chromosomes for an average of 1.2 min (while the chromosome is in antipoleward movement; Skibbens et al., 1993), and the kinetochores spend far more time under tension than under compression (Skibbens et al., 1994). Thus, what looks at first like a difficulty turns out to provide another correlation between the character of the tension-modulated phosphorylation detected with 3F3 and the properties expected of a signal to the checkpoint.

In sum, numerous observations and experiments fit with the proposal that kinetochore protein phosphorylation is the link between tension and the mid-mitosis checkpoint and no observations are in serious disagreement. Additional evidence has just been reported; microinjection of 3F3 antibody into living cells delays the onset of anaphase (Campbell and Gorbsky, 1995).

After the Signal: Missing Links

Even if tension-modulated phosphorylation is the signal to the checkpoint, much remains to be done to link correct chromosome attachment to the exit from mitosis. Important pieces of the puzzle have been discovered: new components and capabilities of the kinetochore (e.g., Brinkley et al., 1992; Earnshaw, 1994; Wang et al., 1994), mutants that affect mitotic checkpoints (Hoyt et al., 1991; Li and Murray, 1991), and biochemical activities associated with cell cycle progression and the exit from mitosis (e.g., Minshull et al., 1994; for review see Murray and Hunt, 1993). But how these pieces fit together is obscure, not least because numerous pieces of the puzzle are missing. Some useful, general speculations have been offered, however (Earnshaw et al., 1991; McIntosh, 1991; Gorbsky, 1995).

One point worth noting is that the signal from the chromosome is not tightly linked to the onset of anaphase. Anaphase onset follows the proper attachment of the last chromosome by tens of minutes in both a mammalian cell line (Rieder et al., 1994) and in insect spermatocytes (Li and Nicklas, 1995; this report). A long delay before anaphase also follows the dephosphorylation of the most tardy kinetochore as detected by 3F3. So the "all clear" signal is sent long before anaphase begins, which implies either a leisurely completion of as yet unidentified final preparations for anaphase or additional controls or checkpoints beyond the one that responds to improper chromosome attachment.

Conclusions

We conclude that tension-modulated protein phosphorylation probably is the signal that permits errors to be detected by linking mitotic mechanics to cell cycle progression. The evidence is circumstantial, a matter of correlating phosphorylation status with checkpoint response. More directly, we show that tension alters chromosome chemistry. As far as we are aware, this is the first strong indication that cellular forces can directly alter the chemistry of a specific (though unidentified) protein. Our studies suggest a transduction of force into chemical change, just the reverse of the more familiar transduction of chemical change into force, as in muscle contraction.

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References

Ault, J. G., and R. B. Nicklas. 1989. Tension, microtubule rearrangements, and the proper distribution of chromosomes in mitosis. *Chromosoma.* 98:33–39.

Bernat, R. L., G. G. Borisy, N. F. Rothfield, and W. C. Earnshaw. 1990. Injection of anticentromere antibodies in interphase disrupts events required for chromosome movement at mitosis. *J. Cell Biol.* 111:1519–1533.

Brinkley, B. R., I. Ouspenski, and R. P. Zinkowski. 1992. Structure and molecular organization of the centromere-kinetochore complex. *Trends Cell Biol.* 2:15–21.

Callan, H. G., and P. A. Jacobs. 1957. The meiotic process in *Mantis religiosa* L. males. *J. Genet.* 55: 200–217.

Campbell, M. S., and G. J. Gorbsky. 1995. Microinjection of mitotic cells with the 3F3/2 antiphosphoepitope antibody delays the onset of anaphase. *J. Cell Biol.* 129:1195–1204.

Cyert, M. S., T. Scherson, and M. W. Kirschner. 1988. Monoclonal antibodies specific for thiophosphorylated proteins recognize *Xenopus* MPF. *Dev Biol.* 129:209–216.

Earnshaw, W. C. 1994. Structure and molecular biology of the kinetochore. *In* Microtubules. J. S. Hyams and C. W. Lloyd, editors. Wiley-Liss, Inc., NY. 392–412.

Earnshaw, W. C., R. L. Bernat, C. A. Cooke, and N. F. Rothfield. 1991. Role of the centromere/kinetochore in cell cycle control. *Cold Spring Harbor Symp. Quant. Biol.* 56:675–685.

Fernandez, A., D. L. Brautigan, and N. J. C. Lamb. 1992. Protein phosphatase type 1 in mammalian cell mitosis: chromosomal localization and involvement in mitotic exit. *J. Cell Biol.* 116:1421–1430.

Goldspink, G., G.-F. Gerlach, T. Jaenicke, and P. Butterworth. 1994. Stretch, overload and gene expression in muscle. *In* Society for Experimental Biology Seminar Series 54: Biomechanics and Cells. F. Lyall and A. J. El Haj, editors. Cambridge University Press, Cambridge, UK. 81–95.

Gorbsky, G. J. 1995. Kinetochores, microtubules and the metaphase checkpoint. *Trends Cell Biol.* 5:143–148.

Gorbsky, G. J., and W. A. Ricketts. 1993. Differential expression of a phosphoepitope at the kinetochores of moving chromosomes. *J. Cell Biol.* 122:1311–1321.

Hartwell, L. H., and T. A. Weinert. 1989. Checkpoints: controls that ensure the order of cell cycle events. *Science (Wash. DC).* 246:629–634.

Hoyt, M. A., L. Totis, and B. T. Roberts. 1991. S. cerevisiae genes required for cell cycle arrest in response to loss of microtubule function. *Cell.* 66:507–517.

Hudspeth, A. J., and P. G. Gillespie. 1994. Pulling springs to tune transduction: adaptation by hair cells. *Neuron.* 12:1–9.

Jordan, M. A., R. J. Toso, D. Thrower, and L. Wilson. 1993. Mechanism of mitotic block and inhibition of cell proliferation by taxol at low concentrations. *Proc. Natl. Acad. Sci. USA.* 90:9552–9556.

Li, R., and A. W. Murray. 1991. Feedback control of mitosis in budding yeast. *Cell.* 66:519–531.

Li, X., and R. B. Nicklas. 1995. Mitotic forces control a cell-cycle checkpoint. *Nature (Lond.).* 373:630–632.

McIntosh, J. R. 1991. Structural and mechanical control of mitotic progression. *Cold Spring Harbor Symp. Quant. Biol.* 56:613–619.

Minshull, J., H. Sun, N. K. Tonks, and A. W. Murray. 1994. A MAP kinase-dependent spindle assembly checkpoint in *Xenopus* egg extracts. *Cell.* 79: 475–486.

Murray, A. 1994. Cell cycle checkpoints. *Curr. Opinion Cell Biol.* 6:872–876.

Murray, A., and T. Hunt. 1993. The Cell Cycle. W. H. Freeman and Company, NY. 251 pp.

Neff, M. W., and D. J. Burke. 1992. A delay in the Saccharomyces cerevisiae cell cycle that is induced by a dicentric chromosome and dependent upon mitotic checkpoints. *Mol. Cell. Biol.* 12:3857–3864.

Nicklas, R. B. 1967. Chromosome micromanipulation. II. Induced reorientation and the experimental control of segregation in meiosis. *Chromosoma.* 21:17–50.

Nicklas, R. B. 1988. Chance encounters and precision in mitosis. *J. Cell Sci.* 89: 283–285.

Nicklas, R. B., and D. F. Kubai. 1985. Microtubules, chromosome movement, and reorientation after chromosomes are detached from the spindle by micromanipulation. *Chromosoma.* 92:313–324.

Nicklas, R. B., and P. Arana. 1992. Evolution and the meaning of metaphase. *J.*

Cell Sci. 102:681–690.

Nicklas, R. B., and S. C. Ward. 1994. Elements of error correction in mitosis: microtubule capture, release, and tension. *J. Cell Biol.* 126:1241–1253.

Nicklas, R. B., B. R. Brinkley, D. A. Pepper, D. F. Kubai, and G. K Rickards. 1979. Electron microscopy of spermatocytes previously studied in life: methods and some observations on micromanipulated chromosomes. *J. Cell Sci.* 35:87–104.

Rieder, C. L. and S. P. Alexander. 1990. Kinetochores are transported poleward along a single astral microtubule during chromosome attachment to the spindle in newt lung cells. *J. Cell Biol.* 110:81–95.

Rieder, C. L., E. A. Davison, L. C. W. Jensen, L. Cassimeris, and E. D. Salmon. 1986. Oscillatory movements of monooriented chromosomes and their position relative to the spindle pole result from the ejection properties of the aster and half spindle. *J. Cell Biol.* 103:581–591.

Rieder, C. L., A. Schultz, R. Cole, and G. Sluder. 1994. Anaphase onset in vertebrate somatic cells is controlled by a checkpoint that monitors sister kinetochore attachment to the spindle. *J. Cell Biol.* 127:1301–1310.

Rogers, K. A., N. H. McKee, and V. I. Kalnins. 1985. Preferential orientation of centrioles toward the heart in endothelial cells of major blood vessels is reestablished after reversal of a segment. *Proc. Natl. Acad. Sci. USA.* 82:3272–3276.

Skibbens, R. V., V. P. Skeen, and E. D. Salmon. 1993. Directional instability of kinetochore motility during chromosome congression and segregation in mitotic newt lung cells: a push-pull mechanism. *J. Cell Biol.* 122:859–875.

Skibbens, R. V., J. C. Waters, and E. D. Salmon. 1994. Oscillating vertebrate kinetochores are on average under tension. *Mol. Biol. Cell. Suppl.* 5:410a.

Sluder, G., F. J. Miller, E. A. Thompson, and D. E. Wolf. 1994. Feedback control of the metaphase-anaphase transition in sea urchin zygotes: role of maloriented chromosomes. *J. Cell Biol.* 126:189–198.

Spencer, F., and P. Hieter. 1992. Centromere DNA mutations induce a mitotic delay in Saccharomyces cerevisiae. *Proc. Natl. Acad. Sci. USA.* 89:8908–8912.

Tomkiel, J., C. A. Cooke, H. Saitoh, R. L. Bernat, and W. C. Earnshaw. 1994. CENP-C is required for maintaining proper kinetochore size and for a timely transition to anaphase. *J. Cell Biol.* 125:531–545.

Wang, X. M., N. Yew, J. G. Peloquin, G. F. Vande Woude, and G. G. Borisy. 1994. Mos oncogene product associates with kinetochores in mammalian somatic cells and disrupts mitotic progression. *Proc. Natl. Acad. Sci. USA.* 91: 8329–8333.

Wendell, K. L., L. Wilson, and M. A. Jordan. 1993. Mitotic block in HeLa cells by vinblastine: ultrastructural changes in kinetochore-microtubule attachment and in centrosomes. *J. Cell Sci.* 104:261–274.

Yen, T. J., D. A. Compton, D. Wise, R. P. Zinkowski, B. R. Brinkley, W. C. Earnshaw, and D. W. Cleveland. 1991. CENP-E, a novel human centromere-associated protein required for progression from metaphase to anaphase. *EMBO J.* 10:1245–1254.

Zirkle, R. E. 1970. Ultraviolet-microbeam irradiation of newt-cell cytoplasm: spindle destruction, false anaphase, and delay of true anaphase. *Radiation Res.* 41:516–537.