INTRODUCTION

Tension plays important regulatory roles in mitosis. When a chromosome is properly attached to the spindle, the partner kinetochores are under tension from forces directed toward opposite poles. Tension, or some consequence of tension, stabilizes the proper spindle attachment (Nicklas and Ward, 1994), controls cell cycle progression (Li and Nicklas, 1995; Waters et al., 1998), causes the dephosphorylation of certain kinetochore proteins (Nicklas et al., 1995), and may regulate force production and/or microtubule assembly at kinetochores (Rieder and Salmon, 1998; Skibbens et al., 1995; Skibbens et al., 1993; Zhai et al., 1996).

It is widely assumed that tension also increases the number of kinetochore microtubules (Rieder et al., 1995; Rieder and Salmon, 1998; Wells, 1996). Apparently this assumption is based on experiments that demonstrate that tension stabilizes chromosome attachment to the spindle (Nicklas and Koch, 1969; Nicklas and Ward, 1994). But attachment stability and changes in kinetochore microtubule number are separate things. Attachment instability is the basis for the correction of attachment errors; improper chromosome attachments are unstable and are lost, while a proper attachment, once attained, persists. Tension applied by a micromanipulation needle stabilizes otherwise unstable, improper attachments. In these experiments stability is measured simply as the length of time a given attachment persists. Persistence time, not microtubule number, is measured. The stability experiments provide no evidence that tension affects the number of kinetochore microtubules.

Another possible source of information on tension and the number of kinetochore microtubules are ‘mono-attached’ chromosomes, in which only one of the two sister kinetochores is attached to the spindle. In the mammalian PtK cell line, the attached kinetochore of such chromosomes were found to have about half as many microtubules as the kinetochores of ‘bi-attached’ chromosomes with kinetochores attached to opposite spindle poles (McEwen et al., 1997). It might be expected that a mono-attached chromosome is not under tension because there is no force from an oppositely directed kinetochore. However, in vertebrate cells tension actually is present: the poleward force at the attached kinetochore is opposed by anti-poleward forces on the chromosome arms, resulting in a visible stretching of the kinetochore region (Waters et al., 1996). So while tension on these kinetochores may be reduced, it is not absent, and the extent of its influence on kinetochore microtubule number in these cells remains ambiguous.

It is important to know whether tension increases the number of kinetochore microtubules because it affects our understanding of the spindle checkpoint. The presence of a single improperly attached kinetochore that is not under tension force of $6 \times 10^{-5}$ dynes, three times the normal tension, on prometaphase kinetochores. The elevated tension did not drive kinetochore microtubule number above normal prometaphase values. Tension probably increases the number of kinetochore microtubules by slowing their turnover rate. The limited effect of tension at prometaphase kinetochores suggests that they have fewer microtubule binding sites than at late metaphase. The relatively few sites available in prometaphase may be the decisive sites whose binding of microtubules regulates the dynamics of transient kinetochore constituents, including checkpoint components.

SUMMARY

When chromosomes attach properly to a mitotic spindle, their kinetochores generate force in opposite directions, creating tension. Tension is presumed to increase kinetochore microtubule number, but there has been no direct evidence this is true. We micromanipulated grasshopper spermatocyte chromosomes to test this assumption and found that tension does indeed affect the number of kinetochore microtubules. Releasing tension at kinetochores causes a drop to less than half the original number of kinetochore microtubules. Restoring tension onto these depleted kinetochores restores the microtubules to their original number. However, the effects of tension are limited. Prometaphase kinetochores, when under normal tension from mitotic forces, have about half as many microtubules as they will in late metaphase. We imposed a tension force of $6 \times 10^{-5}$ dynes, three times the normal tension, on prometaphase kinetochores. The elevated tension did not drive kinetochore microtubule number above normal prometaphase values. Tension probably increases the number of kinetochore microtubules by slowing their turnover rate. The limited effect of tension at prometaphase kinetochores suggests that they have fewer microtubule binding sites than at late metaphase. The relatively few sites available in prometaphase may be the decisive sites whose binding of microtubules regulates the dynamics of transient kinetochore constituents, including checkpoint components.

Key words: Kinetochore, Kinetochore microtubule, Micromanipulation, Spindle checkpoint, Tension

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Tension on chromosomes increases the number of kinetochore microtubules but only within limits
tension activates the checkpoint, which delays the cell cycle (Li and Nicklas, 1995). The effect of tension could be direct; for example, it could deform and inactivate a checkpoint component (Gorbsky, 1995; Nicklas et al., 1998; Nicklas et al., 1995). Alternatively, tension might inactivate the checkpoint indirectly, perhaps by increasing the number of kinetochore microtubules (Nicklas et al., 1995; Rieder et al., 1995; Skibbens and Hieter, 1998; Wells, 1996). Unfilled microtubule binding sites at the kinetochore might have active checkpoint complexes that are displaced or otherwise modified when microtubules bind, thus inactivating the checkpoint and allowing division to proceed. Thus, if tension causes a sufficient increase in kinetochore microtubules to saturate the binding sites, that might be how cells detect when all chromosomes are properly attached to the spindle.

We have used micromanipulation to test directly whether tension affects the number of kinetochore microtubules. It does. Relaxing tension causes a substantial drop in the number of kinetochore microtubules, and restoring the tension restores the normal number. Yet the effects of tension are limited. Greatly increasing tension on prometaphase kinetochores does not increase the number of microtubules beyond the normal number for that stage, even though by metaphase those same kinetochores will have twice as many microtubules. The limited effect of tension on kinetochore microtubule number suggests that the kinetochore’s microtubule binding capacity changes during mitosis and also has implications for how the spindle checkpoint detects improperly attached kinetochores.

MATERIALS AND METHODS

Micromanipulation and live cell observations

Spermatocytes from laboratory colonies of the grasshopper *Melanoplus sanguinipes* (Fabricius) were cultured as previously described (Nicklas et al., 1979) at a temperature of 22.5-25°C. The spermatocytes were viewed by phase contrast microscopy, and their chromosomes were micromanipulated by standard procedures (Nicklas and Ward, 1994 and references therein). Chromosome behavior before, during, and after manipulation was recorded on an optical disk recorder (model 2021, Panasonic Video Systems, Seacaucus, NJ).

Reagents

Pipes/NaCl: 100 mM Pipes (Sigma Chemical Co., St Louis, MO), and 50 mM NaCl (EM Industries, Inc., Gibbstown, NJ), pH 6.8. PBS: 0.1 M NaCl, 0.03 M KCl (Fisher, Fairlawn, NJ), 0.01 M KH₂PO₄ (EM Industries, Inc.), and 0.05 M Na₂HPO₄ (EM Industries, Inc.), pH 7.3. BSA/PBS: 1% bovine serum albumin (Sigma) in PBS.

Electron microscopy

Electron microscopy methods, from fixation to the determination of the number of kinetochore microtubules, were as described earlier (Nicklas et al., 1982; Nicklas and Kubai, 1985).

Fixation and immunostaining

Cells were fixed and bound to the coverslip by micropipetting ‘macrofixative’ near them (Nicklas et al., 1979). The Pipes/NaCl based macrofixative contained either 4% glutaraldehyde (Polysciences, Inc., Warrington, PA) or 4% glutaraldehyde and 2% Chaps (ProChem Inc., Rockford, IL). The unusually high concentration of glutaraldehyde is diluted by the culture fluid bathing the cells. After 3 minutes, the oil covering the cells was flushed off the coverslip with ‘macrofixative’ (1% glutaraldehyde in Pipes/NaCl), and then the coverslips were immersed in macrofixative for 15 minutes (Nicklas et al., 1979). After three 5-minute rinses in PBS, the cells were permeabilized by incubating the coverslips in 0.5% Nonidet P-40 (Sigma) in PBS for 30 minutes at room temperature. Coverslips were then dipped in fresh PBS to rinse off the Nonidet. P-40. To reduce free aldehydes, coverslips were incubated in PBS with 0.5 mg/ml NaBH₄ (Sigma) for 15 minutes. To prevent non-specific antibody binding, the cells were treated with 5% fetal bovine serum (Gibco BRL, Gaithersburg, MD) and 0.2% Tween-20 (Sigma) in PBS for 30 minutes at 37°C. To visualize microtubules, the cells were stained at either 37°C for 45 minutes with the Binder anti-tubulin antibody (TU-27, provided by Lester Binder, University of Alabama, Birmingham, AL) diluted 1:100 in BSA/PBS, or at 5°C for 24 hours with anti-α-tubulin and anti-β-tubulin antibodies (Amersham, Arlington Heights, IL) diluted 1:50 in BSA/PBS. Following three 10-minute rinses in BSA/PBS, cells were labeled with Cy3-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) diluted 1:25 in BSA/PBS. After another three 10-minute rinses in BSA/PBS, coverslips were dipped in distilled water. Coverslips were mounted on slides with either Mounting Medium (0.1 M n-propyl gallate (Sigma) in glycerol (EM Industries, Inc.))/PBS, 1:1, or Vectashield (Vector Laboratories, Burlingame, CA) supplemented with 10 mM CaCl₂. The edges of the coverslips were sealed with nail polish.

Immunofluorescence images and measurements

Immunostained cells were examined with an epifluorescence microscope (Axiovert TV100, Carl Zeiss, Inc., Thornwood, NY) equipped with a Zeiss ×40/1.3 numerical aperture, ICS Plan-Neofluar phase contrast objective; an optivar set to ×2.5; and a cooled CCD video camera (Pentamax model 768-K or Micromax model 1300-Y, Princeton Instruments, Inc., Trenton, NJ). MetaMorph software (Universal Imaging Corp., West Chester, PA) was used to acquire digital images and to measure pixel brightness.

The brightness of immunofluorescent kinetochore microtubule bundles was measured in unprocessed images. A rectangular area having a width slightly wider than a kinetochore microtubule bundle and a length between 0.66 and 0.90 ㎛ was drawn 0.5 ㎛ from the kinetochore. The total brightness of the pixels within the rectangle was measured. This measurement includes the fluorescence of both the kinetochore microtubule bundle and surrounding background. The contribution from background fluorescence was then determined. Lines one pixel in width were drawn on the two long sides of the rectangle. The brightness value per pixel of both lines combined was averaged and then multiplied by the area of the rectangle in pixels, to give the total contribution of background fluorescence within the rectangle. This value was subtracted from the total brightness value of the rectangle, giving a corrected brightness measurement. The corrected brightness was then scaled to represent 1 ㎛ of microtubule bundle length (to compensate for the variable 0.66 to 0.90 ㎛ length of the measurement box). The linearity of the relationship between measured brightness and actual object brightness was verified by measuring the brightness of calibrated neutral density filters. The measured values for filters ranging from 97.7% to 2.3% transmission differed by no more than 3% from the calibrated values. The measurements were made on images at a single focal level with a 1.3 numerical aperture objective. The focal depth of such objectives is great enough to include the fluorescence from the full depth of a kinetochore microtubule bundle; measurements with an objective having a numerical aperture of 0.9 (and therefore substantially greater depth of focus) gave the same value, on average.

For the illustrations in this report, fluorescence and phase contrast images were processed digitally, using commercial software (‘Photoshop’ by Adobe Systems, Inc., Mountain View, CA, and ‘PowerPoint’ by Microsoft Corp., Bellevue, WA). Prints were made with an inkjet printer (970C Series, Hewlett-Packard Co., Corvallis, OR).
RESULTS

Fluorescence images reflect differences in kinetochore microtubule number

Properly attached kinetochores slowly accumulate microtubules throughout the course of prometaphase and metaphase in Melanoplus spermatocytes. Kinetochores in prometaphase of meiosis I have an average of 23 microtubules while those in anaphase have about 45 microtubules, as shown by direct counts from electron micrographs of serially sectioned kinetochores (Nicklas and Kubai, 1985; Nicklas et al., 1982). These differences in microtubule number provide a standard for judging immunofluorescence observations. Immunofluorescence images clearly show the disparity in microtubule number between prometaphase and late metaphase kinetochores: prometaphase kinetochores have thin, dim microtubule bundles (arrowheads, Fig. 1B), while late metaphase kinetochores have thick, bright microtubule bundles (arrowheads, Fig. 1D). As described below, we have confirmed by electron microscopy that immunofluorescence reliably reflects kinetochore microtubule number.

Relaxing tension causes loss of kinetochore microtubules

Immunofluorescence microscopy

Starting with spermatocytes in metaphase of meiosis I, a kinetochore of one chromosome (curved arrow, Fig. 2A,B) was detached from the spindle with a microneedle, but its partner kinetochore (arrow, A,B) remained attached to the spindle. This relaxed the tension on the chromosome, as seen by its immediate shortening (Fig. 2A, inset). The chromosome was maintained in a relaxed state for 15 minutes. Detached kinetochores that face a spindle pole will reattach within 1.6 minutes on average (Nicklas and Ward, 1994). Therefore, to keep the detached kinetochore from reforming a stable attachment to the spindle during the 15 minute interval, the detached kinetochore was frequently nudged with the microneedle. Since the chromosome was no longer under tension, the kinetochore that remained attached to the spindle was no longer constrained and consequently moved closer to its spindle pole. After keeping the chromosome relaxed for 15 minutes, the cell was fixed and stained for microtubules. The kinetochore that remained attached to the spindle in a relaxed state has a thin, weakly staining microtubule bundle (Fig. 2E, arrow) as compared to the thick, bright microtubule bundles of kinetochores that remained under tension (Fig. 2F, arrowheads). For each of eight such experiments, we measured the brightness of the relaxed kinetochore microtubule bundle and of 7 to 13 bundles of unmanipulated chromosomes in the same cell. The brightness of the control bundles that remained under tension varied greatly; on average, there was a threefold difference between the brightest and dimmest bundles. Despite this large range in brightness, in each cell the relaxed kinetochore microtubule bundle was always less bright than any other and therefore had the fewest microtubules. On average, the relaxed kinetochore microtubule bundle was less than half as bright as the average brightness of kinetochore microtubule bundles that remained under tension, and this difference is statistically highly significant (Table 1). One possible explanation of these results is that by chance we chose to manipulate the chromosome with the fewest number of kinetochore microtubules in each cell. The odds of this happening in 8 independent experiments are less than 1 in 162 million ((1/n1)(1/n2)...(1/n8)), where n1 is the number of bundles measured in cell 1, and n2 is the number in cell 2, etc.). The more likely explanation is that loss of tension causes loss of kinetochore microtubules.

Keeping chromosomes relaxed for 15 minutes is long enough for the number of kinetochore microtubules to reach a steady state value: kinetochores that were kept relaxed for 10 minutes (n=3) had a similar loss in microtubule brightness.

We considered the possibility that kinetochore microtubule loss was an effect of the manipulation itself rather than loss of tension: frequently nudging a detached kinetochore for 15 minutes could cause its attached partner to lose microtubules. We positioned chromosomes so that the detached kinetochore was not likely to capture microtubules and needed no further manipulation to prevent it from reattaching, yet its relaxed, attached partner kinetochore still lost microtubules.

Fig. 1. Immunofluorescence images illuminate differences in kinetochore microtubule number. (A and B) Prometaphase cell. (A) Phase contrast image with arrowheads pointing at kinetochores whose microtubule bundles are indicated in B. (B) Corresponding fluorescence image with thin, dim kinetochore microtubule bundles (arrowheads). (C and D) Late metaphase cell. (C) Phase contrast image with arrowheads pointing at kinetochores whose microtubule bundles are indicated in D. (D) Corresponding fluorescence image with thick, bright kinetochore microtubule bundles (arrowheads). Bar, 10 μm.
The same experiment described above was done in two metaphase cells, only this time not one, but two chromosomes in each cell were relaxed for 15 minutes. The cells were then fixed and processed for electron microscopy. In cell 1, the two relaxed kinetochores had microtubule counts of 13 and 20, respectively, while 6 control kinetochores that were under tension from spindle forces had an average of 40 microtubules (range 27-55). The relaxed kinetochore with 20 microtubules and a control kinetochore with 40 microtubules are shown in Fig. 3. In cell 2, the two relaxed kinetochores had microtubule counts of 8 and 11, respectively, while 8 control kinetochores that had been under tension had an average of 24 microtubules (range 11-39). These counts verify that relaxed kinetochores have many fewer microtubules than kinetochores that remained under tension. They also show that control bundles can have a 3-fold range in number of microtubules. In these two cells the four relaxed kinetochores had on average 40% as many microtubules as control microtubules that remained under tension (Table 1).

Then we restored tension on the relaxed kinetochore (arrow, Fig. 4C) by pulling on the chromosome with a microneedle – note the attenuation in the arm above the arrow (compare with Fig. 4B). After keeping the kinetochore under tension for 15 minutes, the cell was fixed and stained for microtubules. The kinetochore that had been under needle tension (arrow, Fig. 4C,D) and the kinetochores that remained under normal mitotic tension (arrowheads, Fig. 4C,D) have bundles of similar size and brightness. This result was obtained consistently in a set of six experiments.

For each experiment, the brightness of the kinetochore microtubule bundles (E and F) was compared to the brightness of control microtubule bundles (D) to determine the percentage of microtubules lost during relaxation. The brightness of the relaxed kinetochore microtubule bundle was calculated by dividing the number of microtubules in the relaxed bundle by the number of microtubules in the control bundle. The results are shown in Table 1.

![Fig. 2. Relaxing tension causes kinetochore microtubule loss. Upper row: Phase contrast images. (A and B) Live cell images. One kinetochore of a chromosome (lower, curved arrow) was detached from spindle microtubules and kept detached for 15 minutes. During this time its partner kinetochore (upper arrow) remained attached but relaxed, as indicated by the shortening and thickening of chromosome arms. Inset shows the chromosome in the presence (left) or absence (right) of tension. (C and D) Fixed cell images. (C) The arrow points to the attached, relaxed kinetochore and the position it occupied 15 minutes after its partner kinetochore was detached. (D) The arrowheads indicate unmanipulated kinetochores in the same cell but at a different focal level. Lower row: Microtubule immunofluorescence. (E and F) Fixed cell images corresponding to C and D. The relaxed kinetochore microtubule bundle (E, arrow) has fewer microtubules than microtubule bundles under tension (F, arrowheads). Bar, 5 μm.](image)

### Table 1. Brightness comparisons of kinetochore microtubule bundles

<table>
<thead>
<tr>
<th>Cell</th>
<th>Relaxed avg. control</th>
<th>Reapplied tension avg. control</th>
<th>High tension avg. control</th>
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<tr>
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<td>0.26</td>
<td>0.60</td>
<td>0.91</td>
</tr>
<tr>
<td>2</td>
<td>0.32</td>
<td>0.79</td>
<td>1.30*</td>
</tr>
<tr>
<td>3</td>
<td>0.42</td>
<td>1.33</td>
<td>1.06*</td>
</tr>
<tr>
<td>4</td>
<td>0.61</td>
<td>0.97</td>
<td>0.76</td>
</tr>
<tr>
<td>5</td>
<td>0.28</td>
<td>1.07</td>
<td>1.61</td>
</tr>
<tr>
<td>6</td>
<td>0.36</td>
<td>1.19</td>
<td>0.78</td>
</tr>
<tr>
<td>7</td>
<td>0.52</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>0.28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Avg.</td>
<td>0.38</td>
<td>0.99</td>
<td>1.07</td>
</tr>
<tr>
<td>Std dev.</td>
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<td>0.26</td>
<td>0.33</td>
</tr>
<tr>
<td>P</td>
<td>0.0003</td>
<td>0.973</td>
<td>0.857</td>
</tr>
</tbody>
</table>

*P is the significance of the difference between the experimental population and a population which supports the null hypothesis. The significance was tested using a 2-tailed t-test.

*These two kinetochores were in the same cell.
Fig. 3. Kinetochore microtubule counts by electron microscopy confirm that loss of tension leads to loss of kinetochore microtubules. (A) Phase contrast images of the cell in life showing successively the cell just before the operation, immediately after detachment of one kinetochore (curved arrow), and 9.4 minutes later. Arrow: the relaxed kinetochore; arrowhead: a control kinetochore. The cell was fixed 15 minutes after the detachment. (B) Survey electron micrograph with boxes showing the areas in C and D. Bar in B, 10 μm (for A and B). (C and D) Three serial sections from the middle of the control kinetochore (C) and from the relaxed kinetochore (D) from the total of 8 sections through each kinetochore. Kinetochore microtubules as identified at higher magnification are traced in red. Bar in D, 0.5 μm (for C and D).
microtubule bundle that had been under needle tension was compared to the brightness of 5 to 10 control bundles under tension from normal mitotic forces. Invariably, the brightness of the bundle under needle tension was within the range of values observed for control bundles. The brightness of bundles under needle tension was on average 0.99 (range 0.6-1.3) of those associated with unmanipulated chromosomes in the same cell, and the means for the needle and control bundles are statistically indistinguishable (Table 1). Thus, when tension is applied to kinetochores with a paucity of microtubules, the number of microtubules increases to about the level found in chromosomes under tension from normal mitotic forces.

High tension does not cause kinetochores to become fully loaded with microtubules
Prometaphase chromosomes have fewer kinetochore microtubules than late metaphase chromosomes. Will applying tension greater than the normal mitotic tension cause the number of microtubules at prometaphase kinetochores to increase toward the ‘fully loaded’ condition seen at late

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**Fig. 4.** Tension increases microtubule number at previously relaxed kinetochores. (A-C) Live cell images. A metaphase kinetochore (upper, curved arrow) was detached from spindle microtubules with a microneedle and kept from reattaching for 15 minutes. During this time its partner kinetochore (lower arrow) was relaxed but remained attached. Then tension was restored to this kinetochore by pulling on the chromosome with a microneedle for 15 minutes. The arrowheads in C point to unmanipulated kinetochores whose microtubule bundles are shown in D. (D) Microtubule immunofluorescence. The kinetochore microtubule bundle of the kinetochore to which tension was reapplied (arrow) is as bright as the unmanipulated microtubule bundles that were under tension from mitotic forces throughout the experiment (arrowheads). Bar, 10 μm.

**Fig. 5.** Tension has a limited influence on kinetochore microtubule number. Upper row: Live cell images. (A) Kinetochores under normal spindle tension. Arrows mark the kinetochores of the chromosome that will be stretched by a microneedle. (B) By pulling on the chromosome with a microneedle, kinetochores (arrows) were placed under high tension for 15 minutes; note the resulting elongation of the chromosome arms. (C and D) Immediately after microneedle is withdrawn. Arrows indicate the kinetochores that had been under high tension, and arrowheads point to control kinetochores. Lower row: Fluorescence images. (E and F) Kinetochore bundles that had been under high tension (arrows) are no brighter than control bundles which were under normal spindle tension (arrowheads). Bar, 10 μm.
metaphase? To answer this question we used early to mid metaphase I chromosomes that were under tension from normal mitotic forces (arrows, Fig. 5A) but that had not yet developed the higher numbers of kinetochore microtubules characteristic of late metaphase (compare the bundle fluorescence in Fig. 5E,F with that in Fig. 1D). Using a microneedle, we stretched a chromosome (Fig. 5B) for 15 minutes. Then the cell was fixed and stained for microtubules. The kinetochore bundles that had been under high tension were similar in size and brightness (arrows, Fig. 5E) to bundles that had been under normal mitotic tension (arrowheads, Fig. 5E,F). Similar results were obtained for a total of six manipulated kinetochore microtubule bundles in five cells.

The force applied via a microneedle can be measured in absolute units, because the elastic constant of these chromosomes has been determined (Nicklas, 1983; Nicklas, 1988). The tension force, F, placed on a chromosome by either a microneedle or the normal mitotic forces is equal to \( k(l_o - l_o)/l_o \), where \( k \) is the elastic constant, \( 7.0 \times 10^{-3} \); \( l_o \) is the chromosome length under tension, and \( l_o \) is the original, relaxed chromosome length. We did not have measurements of the original, relaxed length of the chromosomes, so we estimated them from measurements of the average mitotic force on prometaphase chromosomes, \( 2.1 \times 10^{-5} \) dynes (average mitotic force on 10 chromosomes, each from a different prometaphase cell). The average force from the micromanipulation needle was \( 6.1 \times 10^{-5} \) dynes, which is 2.9 times greater (range 2.2-3.7) than the normal mitotic forces on the kinetochores.

The brightness of kinetochore microtubule bundles that had been exposed to experimentally elevated tension was measured and compared with the brightness of 8 to 10 control bundles in the same cell. The kinetochore bundles exposed to high tension were never brighter than the brightest control bundle. Considering all experiments together, there was no measurable difference between kinetochore microtubule bundles under artificially high tension and bundles under normal mitotic tension: the average ratio of the brightness of bundles subjected to high tension to the average brightness of controls was 1.1; the means are statistically indistinguishable (Table 1). Even kinetochores that were under high tension for 25 minutes \((n=3)\) were indistinguishable from control kinetochores. Clearly, tension can only increase the number of kinetochore microtubules to the maximum characteristic of a particular stage in mitosis.

**DISCUSSION**

**Tension and kinetochore microtubule number**

Our experiments in living insect cells in meiosis directly show that tension affects kinetochore microtubule number. Relaxing the tension at kinetochores leads to a decrease in the number of kinetochore microtubules, and adding tension back leads to an increase in that number, but only up to the number normally present. Tension may also affect kinetochore microtubules in vertebrate cells in mitosis, providing at least a partial explanation for the reduced number at mono-attached chromosomes observed by McEwen and co-workers (McEwen et al., 1997). It has been assumed that tension increases the number of kinetochore microtubules (e.g. Rieder and Salmon, 1998; Wells, 1996), but until now there was no clear evidence that this is so.

How might tension cause an increase in kinetochore microtubule number? Kinetochore microtubules are relatively stable, but nevertheless they do turn over slowly (Zhai et al., 1995). Hence, the number of microtubules present at steady state reflects a balance between the rate of capture of new kinetochore microtubules and the rate of loss of old ones. Tension might increase the rate of capture by increasing the number of available microtubule binding sites. The kinetochore is visibly pulled out when tension is present (Skibbens et al., 1993), and this might expose previously hidden binding sites. However, any such effect must be limited because unattached prometaphase kinetochores quickly capture microtubules (Nicklas and Ward, 1994; Rieder and Alexander, 1990) despite the absence of tension. The major effect of tension probably is to decrease the rate of loss of existing kinetochore microtubules. A labile linkage between the kinetochore and each kinetochore microtubule would lead to the occasional release of a microtubule, providing an explanation for microtubule turnover (Zhai et al., 1995; Zhai et al., 1996). Motor proteins are prime candidates for maintaining the kinetochore-microtubule link (Lombillo et al., 1995; reviewed by Grancell and Sorger, 1998), and their grip on the microtubule might well be stronger under tension. For example, tension might cause the motors to stall in a tight-binding state (McIntosh and Hering, 1991). If so, upon release of tension, as in our manipulation experiments, the stalled motors would undergo a conformational change and complete their motor cycle, releasing the microtubules. An interesting alternative, or adjunct, to a tension-sensitive attachment is the kinesin-like protein XKCM1. XKCM1 is found at Xenopus centromere regions and spindle poles (Walczak et al., 1996). It uses energy from ATP hydrolysis to depolymerize microtubules, most likely by physically disrupting microtubule ends (Desai et al., 1999). Tension could regulate XKCM1 activity by deforming the structure of kinetochores or spindle poles, making the XKCM1 less accessible to microtubules.

**Tension and proper chromosome attachment**

Earlier work established that tension enhances the stability of chromosome attachment to the spindle (Nicklas and Ward, 1994). This effect of tension is probably distinct from the effect on kinetochore microtubule number described in this report. For attachment stabilization, the best bet is that tension promotes a more stable attachment of kinetochore microtubules at their poleward ends, not at the kinetochore (for discussion see Nicklas and Ward, 1994). The release of microtubules from their polar attachments is now well documented (e.g. Belmont et al., 1990; Hartman et al., 1998; Keating et al., 1997), and tension may inhibit that release. It is possible that tension stabilizes the linkage of kinetochore microtubules at both ends: the polar effect may stabilize chromosome attachment while the kinetochore effect may increase the number of kinetochore microtubules. The molecular complexes at the pole and the kinetochore are now being characterized (Hartman et al., 1998; reviewed by Dobie et al., 1999; Merdes and Cleveland, 1997; Rieder and Salmon, 1998). A direct demonstration that tension enhances the stability of these complexes is the next challenge.

Even though there may be distinct mechanisms by which...
tension stabilizes attachments and increases kinetochore microtubule number, an increase in microtubule number would contribute to attachment stability. Improper attachments are maintained by several kinetochore microtubules that tether the kinetochore to the pole. They must be lost if a new, proper connection to the opposite pole is to be formed, and direct tests show that a misattached kinetochore remains tethered to the pole more than half the time (Nicklas and Ward, 1994). When tension causes a doubling of the number of kinetochore microtubules, there is a greatly decreased probability that all microtubules will come loose at the same time. Hence an increased number of kinetochore microtubules should reinforce the stability of proper attachments.

Tension and kinetochore dynamics

The limits of tension’s effects on kinetochore microtubule number are as significant as the effects themselves. In grasshopper spermatocytes, prometaphase kinetochores of properly attached chromosomes have only half as many kinetochore microtubules as late metaphase kinetochores (Nicklas and Kubai, 1985; Nicklas et al., 1982). We attempted to increase the number of microtubules associated with prometaphase kinetochores by increasing the force to 6x10−5 dynes, three times the normal force, but our attempts failed. Hence the normal increase in number seen from prometaphase to metaphase is not due to an increase in tension. The most likely alternative is that the number of binding sites for kinetochore microtubules increases during mitosis. On this view, properly attached prometaphase kinetochores with 20 microtubules are saturated: all available binding sites are occupied and greater tension cannot increase microtubule number. The later increase to about 45 microtubules results from the creation or unmasking of additional binding sites. Consistent with this, the rate at which kinetochores acquire microtubules increases throughout metaphase (McEwen et al., 1997).

Kinetochore saturation as a stage-specific property would help explain the dynamics of certain kinetochore proteins and spindle checkpoint regulation. There is a growing list of proteins which are abundant at prometaphase kinetochores and then are depleted or absent from metaphase kinetochores, including dynein (Pfarr et al., 1990; Steuer et al., 1990), dynactin (Echeverri et al., 1996), Mad2 (Waters et al., 1998), CLIP-170 (Dujardin et al., 1998), ZW10 (Williams et al., 1996), CENP-E (Thrower et al., 1996), and MAP kinase (Shapiro et al., 1998). Of these proteins, the kinetochore binding dynamics of the spindle checkpoint protein Mad2 are the best characterized. The spindle checkpoint delays the cell cycle in response to unattached or improperly attached kinetochores. Mad2 is abundant on unattached kinetochores and dissociates from the kinetochore as microtubules bind. It is absent from prometaphase kinetochores although by late metaphase standards these kinetochores are only half saturated with microtubules (McEwen et al., 1997; Waters et al., 1998). Our interpretation is that the prometaphase kinetochores are completely saturated with microtubules; i.e., all microtubule binding sites available at that time are filled, and thus Mad2 dissociates completely. This suggests that only the prometaphase sites matter: when they are filled, the ‘wait’ checkpoint signal is lifted and the microtubule binding sites that become available later are irrelevant to checkpoint operation. The binding and release of many potentially potent kinetochore proteins may be regulated only by the subset of microtubules that are bound to the kinetochore during prometaphase.

In conclusion, the effect of tension on kinetochore microtubule number poses interesting questions about the mechanism of tension’s effect and why the effect is limited. The answers to these questions will bear on the binding and release of both kinetochore microtubules and transient kinetochore proteins such as motors and checkpoint constituents.

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REFERENCES


Tension increases kinetochore microtubules