Kinetic analysis of mitotic spindle elongation in vitro

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Summary

Studies of mitotic spindle elongation in vitro using populations of diatom spindles visualized with immunofluorescence microscopy have shown that the two interdigitating half-spindles are driven apart by an ATP-dependent process that generates force in the zone of overlap between half-spindles. To characterize further the system responsible for spindle elongation, we observed spindle elongation directly with polarized light or phase-contrast video-microscopy. We report that the kinetics of spindle elongation versus time are linear. A constant rate of spindle elongation occurs despite the continuous decrease in length of the zone of overlap between half-spindles. The average rate of spindle elongation varies as a function of treatment, and rates measured match spindle elongation rates measured in vivo. When spindles elongated in the presence of polymerizing tubulin (from bovine brain), the extent of elongation was greater than the original zone of half-spindle overlap, but the rate of elongation was constant. No component of force due to tubulin polymerization was found. The total elongation observed in the presence of added tubulin could exceed a doubling of original spindle length, matching the elongation in the intact diatom. The linear rate of spindle elongation in vitro suggests that the force transducer for anaphase B is a mechanochemical ATPase, analogous to dynein or myosin, and that the force for spindle elongation does not arise from stored energy, e.g. in an elastic matrix in the midzone. Additionally, on the basis of observations described here, we conclude that the force-transduction system for spindle elongation must be able to remain in the zone of microtubule overlap during the sliding apart of half-spindles, and that the transducer can generate force between microtubules that are not strictly antiparallel.

Key words: anaphase B, diatom spindle, mechanochemical ATPase, microtubule sliding, mitosis in vitro.

Introduction

The separation of chromosomes at mitosis is an example of a process in which the cell transforms chemical energy into work. Chromosome movement has two components, chromosome-to-pole movement (anaphase A) and spindle elongation (anaphase B) (Inoué and Ritter, 1975). The molecular mechanisms that drive either type of chromosome movement have not yet been discovered (Inoué, 1981; McIntosh and Koonce, 1989; Mitchison, 1989; Nicklas, 1988). In contrast to chromosome movement, muscle contraction and ciliary beat are powered by well-characterized mechanochemical enzymes, myosin for muscle and dynein for cilia (Bagshaw, 1982; Gibbons, 1981). Each of these molecular motors has been modeled as a cycling crossbridge (Harrington and Rogers, 1984; Johnson, 1985). The cycling crossbridge model has also been applied to other intracellular motors, myosin I (Korn and Hammer, 1988) kinesin (Scholty et al. 1989) and cytoplasmic dynein (Shpetner et al. 1988). It is therefore natural to assume that a cycling crossbridge analogous to dynein or myosin is responsible for spindle elongation; but there is no evidence to rule out other mechanisms, for example, the release of energy stored in an elastic matrix (McIntosh, 1981; Cande and Hogan, 1989; Mitchison, 1989). Furthermore, the kinds of motility known to be driven by cycling crossbridges occur at rates several orders of magnitude faster than the rate of spindle elongation, which could indicate that the functional organization of the force-generating system is other than a cycling crossbridge. Another type of slow (i.e. μm min⁻¹) microtubule-based intracellular motility is apparently driven by an unusual gelation-contraction mechanism (Gao and Wisenberg, 1988).

An advance toward identifying the mechanism that drives anaphase B came from the development by Cande and McDonald (1985) of a system in which mitotic spindles isolated from a diatom would elongate in vitro when treated with ATP. This system allowed the mitotic spindle for the first time to be accessible directly to experimentation, and this led to several findings. The zone of microtubule overlap between half-spindles is the site where force is generated for spindle elongation (Cande and McDonald, 1985), incorporation of exogenous tubulin into the spindle apparently does not contribute to force production for elongation (Masuda and Cande, 1987; Masuda et al. 1988), and the pharmacology of spindle elongation resembles that of cytoplasmic dynein (Cande and Hogan, 1989; Shpetner et al. 1988). The similarity of the anaphase B motor to dynein indicated by the pharmacological data is contrasted with the difference in polarity; spindle elongation is an example of plus-end-directed microtubule motility, which is the opposite to that found for dyneins.
(with the exception of a dynein-like enzyme in Reticulomyxa that causes bi-directional movement; Euteneuer et al. 1989).

To extract more information from the diatom spindle working in vitro, it is necessary to make direct observations and study the kinetics of spindle elongation. Direct observation has played a definitive role in the successful study of the contraction of isolated muscle fibers (Huxley, 1977), the beating of demembranated cilia (Gibbons, 1981) and the movement of microtubules over a surface coated with kinesin (Scholey et al. 1989). Biochemical investigations of spindle-associated proteins have been difficult (Inoue, 1981; Wordeman and Cande, 1987) but can be aided if linked to results from direct observation of spindle motility. Knowing the function of spindle elongation will help identify the kind of force-transduction system that drives anaphase B, and will constrain models based on subsequently gathered biochemical data. Baskin and Cande (1988) developed conditions where spindle elongation could be reliably observed in real time with video microscopy. We use this method here to characterize the kinetics of spindle elongation. We report that the rate of spindle elongation is linear under all circumstances, including elongation in elongation. We report that the rate of spindle elongation is linear under all circumstances, including elongation in elongation. We report that the rate of spindle elongation is linear under all circumstances, including elongation in elongation. We report that the rate of spindle elongation is linear under all circumstances, including elongation in elongation. We report that the rate of spindle elongation is linear under all circumstances, including elongation in elongation. We report that the rate of spindle elongation is linear under all circumstances, including elongation in elongation.

**Materials and methods**

**Cell growth and spindle isolation**

Cultures of the marine, centric diatom, *Stephanopyxis turris* (strain L1272), were obtained from the Culture Collection of Marine Phytoplankton (Bigelow Laboratory for Ocean Sciences, West Boothbay, ME), and were grown under a 12:12 photoperiod, in nutrient-supplemented sea water as described by Masuda and Cande (1987).

The procedure for isolating mitotic spindles from cells was similar to that of Baskin and Cande (1987). Essentially, 20 mM nocodazole was added to cell cultures at a specific time in the photoperiod, and washed out 2.5 h later. After 25 min, cells were washed once in ice-cold, Ca^2+/-free, isotonic medium and then homogenized on ice with three strokes of a glass tissue grinder. The homogenate was passed through a 15 µm Nitex mesh and then layered on a 50% sucrose cushion and spun at 4°C for 20 min at 2500 rpm. Sedimenting nuclei, 10–30% of which contained mitotic spindles, were collected on half-coverslips (12 mm diameter, 0 thickness). Half-coverslips were frozen in a cryoprotectant solution in liquid nitrogen.

Two different buffers were used in isolations. For some preparations, the homogenization buffer consisted of 50 mM Pipes (pH 7.0), 8 mM EDTA, 0.5 mM spermine, 1 mM spermidine, 30% glycerol, 0.2% Brij 58 (Polyoxyethylene 20 cetyl ether, from Sigma), 10 mM dithiothreitol (DTT), 1 mM sodium azide, 0.1 mM Trolox (rac-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid); from Fluka, Ronkonkonkoma, NY), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and a set of proteolysis inhibitors as given by Masuda and Cande (1987). The sucrose cushion was layered on a 50% sucrose cushion solution in liquid nitrogen.

The base buffer for experimental treatments contained 2 mM MgSO₄, 5 mM EGTA, 40 mM β-glycerophosphate, 1 mM azide, 0.1 mM Trolox, 10 mM DTT and the proteolysis inhibitors. For mounting, buffer included 75 mM Hepes (pH 7.5) and 50 mM ATP:Si (adenosine-5'-O-(3-thiotriphosphate)); for reaction, buffer included 1 mM ATP and 20 mM Pipes (pH 7.0) or 75 mM Hepes (pH 7.5). For DNase treatment, DNase I (type II from Sigma) was added to the mounting media, with DTT reduced to 0.5 mM.

Bovine brain tubulin was prepared as described by Masuda and Cande (1987) and was substantially free of contaminating microtubule-associated protein. For experiments with tubulin addition, spindles were digested briefly with DNase to remove most of the surrounding chromatin. Then a solution of tubulin and GTP was mixed with one containing taxol and ATP:Si, or for reactivation, ATP, and immediately perfused into the chamber. The final concentration of GTP was 0.5 mM; taxol, 20 µM; ATP:Si, 50 µM; and tubulin 1–2 mg/ml as noted; other components were as given for reactivation medium (pH 7.0) above. Controls were run by omitting the tubulin.

**Microscopy**

A Zeiss Standard stand was used. For illumination, light from a high-pressure mercury arc lamp (HBO 100 W bulb) passed through two heat filters (KG-3, 3 mm thick, Melles Griot, Irvine, CA) and a narrow-pass, interference filter, transmitting maximally at 546 nm. For phase-contrast, a neofluor (40×, 0.75 NA) objective was used; for polarized light, a plan (40×, 0.8 NA) objective was used. For polarized light, a condenser numerical aperture of 0.6 was used. The compensator port was modified to accept a custom-built compensator, comprising a device to hold and rotate a 1 inch diameter retainer and give a digital readout of the angle, and either a X/30 quartz retainer (Karl Lambrecht, Chicago, IL) or a X/10 retainer made from an organic polymer (Meadowlark Optics, Longmont, CO). For video recording, a bias compensation of roughly 15 nm was usually used.

The microscope image was projected via an ocular to the video camera faceplate approx. 15 cm distant from the ocular. The signal from a video camera (Series 67M, Dage/MTI, Michigan City, IN) with a Newvonix tube, and manual gain and black level control was passed through a time/date generator (WJ-500, Panasonic) and was recorded in real time in the Super-VHS format with a VCR (HRS7000-U, JVC). Micrographs for publication were prepared by playback of the videotape through a digital image processor (Image 1/AT, Universal Imaging Co., Media, PA) and 8-frame averages stored at appropriate times.

Contrast was enhanced as needed, and negatives were made with a slide maker (Montage FB1, Freamont, CA). The line scans of image grey level (see Fig. 4) were made by playback of the videotape through the image processor, without digital enhancement, using a utility of the processor to graph the average intensity along a strip, set at a width somewhat less than the spindle width.

**Quantification**

Quantitative information was recovered by playback of the...
videotape through an image analysis system comprising a computer (IBM-compatible AT), a frame grabber (PC Vision Plus board, Image Technology, Woburn, MA) and software (MorphoSyx, from Drs C. Meacham and T. Duncan, University of California Herbarium, Berkeley, CA). Lengths were measured on frozen video frames by manipulating a cursor, with neither a numerical nor a graphical representation of the measured length being visible to the measurer. For each timepoint, four replicate measurements of spindle length were made from a single frozen video frame, with the average being used for subsequent analysis. The standard deviation of these replicates was typically 0.1 μm (range: 0.02–0.2 μm), which was about the size of a single pixel. Although measurement with the light microscope of the size of an object cannot be accurate if the size of that object falls below the diffraction-based resolution limit (about 0.5 μm for the present optics), measurement of the size difference between two well-focused objects (e.g. the spindle poles, situated about 10 μm apart) is not subject to this resolution limit, and is limited instead by image characteristics and the spatial frequency of sampling the image (Inoué, 1986; Gelles et al. 1988). Therefore, the measurement precision reported here does not conflict with the microscope’s resolving power.

Spindle lengths were measured at known times; in many instances, measurements were made out of the natural time sequence to minimize experimenter bias. To quantify rates of spindle elongation, a linear regression line was fitted to the data points for which elongation was clearly occurring. The number of points was typically 12 (range 6–28).

In recordings with polarized light, neither camera controls nor compensator setting was altered during the videotaping; therefore the grey level of a pixel in the object reports the birefringent retardation (referred to as retardation in the following) of that location in the object. To measure the grey level for regions of interest in the spindle, we obtained the average grey level during one video frame for all pixels in a rectangular box, which could be sized and positioned at will. Readings were taken for background (approx. 1000 pixels) areas adjacent to the spindle, for a region in the middle of the spindle, and for one halfway between the spindle pole and the apparent edge of the zone of microtubule overlap. The regions spanned nearly the full spindle width and were 2 pixels in length (approx. 0.2 μm). At each timepoint, the background grey level was subtracted from the spindle grey level, which results in negative grey levels for spindle regions but fluctuations in light intensity are removed. The relation between specimen retardation and intensity of light in its image is not linear (Hiramoto et al. 1988). Therefore, the measurement procedure of the present study does not conflict with the microscope’s resolving power.

Results

The kinetics of spindle elongation

The kinetics of spindle elongation versus time are linear (Fig. 1). Linear kinetics were observed with phase-contrast or polarized light optics. Onset of elongation is difficult to measure exactly because spindles usually begin to elongate during perfusion while being jostled by the turbulent flow of solution in the chamber. The appearance at the start of the time course in Fig. 1 of a slight shrinkage of the spindle is due to perfusion; in other examples, abrupt enlargements are seen. The lag time from the start of ATP perfusion to the beginning of elongation varies from several seconds to several minutes, apparently at random. The final 10–20% of the elongation time course sometimes shows a period of deceleration (Fig. 1). This deceleration may be real or may result from movement of a half-spindle slightly out of the focal plane and the consequent underestimation of spindle length. Examples where one half-spindle moved totally out of the focal plane were common and were not analysed. Examples were also observed where bending apparently occurred entirely within the plane of focus; decelerations in the final part of the time course were not always observed in such examples.

To confirm the linearity of the kinetics, we fitted linear regression lines to the data, over that part of the time course when elongation occurred. Table 1 shows representative data for one treatment. In each example, the standard error of the slope (i.e. of the regression coefficient) is typically 10% or less of the value of the slope, indicating a good fit to a line. Furthermore, a slope drawn through the data by hand was always within 10% of the regression coefficient. Analysis of the residual deviations of the data from the regression line for 45 examples failed to provide evidence for a departure from a zero average residual over any time interval during elongation (not shown); this rules out the presence of a systematic departure from the linear nature of the kinetics within measurement precision. Finally, in all, 115 spindles have been measured without observing an example where the rate of elongation appeared to decelerate (or accelerate) continuously. In these experiments, under various treatments, linear kinetics occur over a range of magnitude of

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**Table 1. Regression analysis of the rate of isolated spindle elongation**

<table>
<thead>
<tr>
<th>Example</th>
<th>Regression coefficient* (μm min⁻¹)</th>
<th>Standard error† (μm min⁻¹)</th>
<th>Number of points</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.6</td>
<td>0.05</td>
<td>11</td>
</tr>
<tr>
<td>2</td>
<td>1.4</td>
<td>0.14</td>
<td>8</td>
</tr>
<tr>
<td>3</td>
<td>0.9</td>
<td>0.07</td>
<td>10</td>
</tr>
<tr>
<td>4</td>
<td>0.5</td>
<td>0.04</td>
<td>13</td>
</tr>
<tr>
<td>5</td>
<td>1.2</td>
<td>0.14</td>
<td>10</td>
</tr>
<tr>
<td>6</td>
<td>1.1</td>
<td>0.13</td>
<td>9</td>
</tr>
<tr>
<td>7</td>
<td>1.1</td>
<td>0.2</td>
<td>13</td>
</tr>
<tr>
<td>8</td>
<td>1.0</td>
<td>0.05</td>
<td>14</td>
</tr>
<tr>
<td>9</td>
<td>0.6</td>
<td>0.05</td>
<td>14</td>
</tr>
<tr>
<td>10</td>
<td>0.8</td>
<td>0.05</td>
<td>10</td>
</tr>
<tr>
<td>Average</td>
<td>0.9</td>
<td>0.08</td>
<td>11.2</td>
</tr>
</tbody>
</table>

*That is, the slope of the regression line.
† Standard error of the regression coefficient.

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**Fig. 1. Spindle length versus time for elongation of an isolated spindle reactivated at pH 7.0.** Note the approximate linearity of elongation. At t=0, 1 mM ATP was added. Symbols in this and all subsequent time-course graphs plot the mean of four replicate measurements, and lines were drawn through the data points by hand. All Figs 1, 3, 6 and 8 are from polarized light records unless noted otherwise.

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**Kinetics of anaphase B in vitro**

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Time = l min, 1 mM ATP was added. Midzone length is defined the rates of spindle elongation and midzone shrinkage. At
viewed with phase-contrast. Note the approximate linearity of
Fig. 2. Spindle length and midzone length versus time for
 elongation of an isolated spindle reactivated at pH 7.5 and
and methods.
Isolated spindles are usually enveloped by a cloud of
chromatin (Baskin and Cande, 1988). To determine
whether this material affected the elongation kinetics, Dnase I
digestions were allowed to run to completion before ATP
perfusion. Complete digestion was inferred in
two ways: first, by the total disappearance of phase-dense
material that surrounds the spindle; and second, by the
ability of spindles after several minutes incubation in
DNase to float freely through the perfusion chamber,
eventually settling on the glass substratum to which they
adhered. The kinetics of spindle elongation after DNase
digestion are linear (Fig. 3).
For PESS homogenization, there is no difference in the
mean rate of elongation with or without DNase digestion
(Table 2). Therefore, the cloud of surrounding chromatin
has no required role in spindle elongation in vitro.
Spindles that have been homogenized and frozen in PESS
homogenization buffers was tested. Because the
cloud of surrounding chromatin had no required role in spindle
elongation at pH 7.0 (but not at pH 7.5), which
equals the rate measured for PESS-homogenized material
(Table 2). No explanation for the differences between
homogenization buffers was tested. Because the
 elongation rate obtained at pH 7.0 with PESS homogenization
is within the range of those measured for this diatom
in vivo (McDonald et al. 1986), we have used this
homogenization buffer for the subsequent experiments on tubulin
incorporation.

**Behavior of the overlap zone during spindle elongation**

The zone of microtubule overlap between half-spindles is
usually visible in both phase-contrast and polarized light
observation (Baskin and Cande, 1988). In the absence of
exogenous tubulin, the extent of spindle elongation equals
the initial size of the overlap zone (Table 3). This shows
that the movement of half-spindles depends on force
generated within the zone of microtubule overlap, and
rules out the involvement of force production at other
sites, for example, from microtubule-translocating proteins
that had become bound to the glass substratum.

The overlap zone begins to shrink as soon as the spindle
begins to elongate, and the kinetics of midzone shortening
are approximately linear (Fig. 2), although measurements
of overlap zone length are less certain than those of spindle

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**Table 2. Rates of isolated spindle elongation as a function of treatment**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Rate ± s.e.m. (μm min⁻¹)</th>
<th>(n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PME homogenization*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 7.0</td>
<td>0.9 ± 0.09</td>
<td>(10)</td>
</tr>
<tr>
<td>+ Partial DNase†</td>
<td>1.3 ± 0.3</td>
<td>(9)</td>
</tr>
<tr>
<td>pH 7.5</td>
<td>0.4 ± 0.03</td>
<td>(9)</td>
</tr>
<tr>
<td>+ Partial DNase†</td>
<td>0.4 ± 0.05</td>
<td>(8)</td>
</tr>
<tr>
<td>PESS homogenization*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 7.0</td>
<td>1.8 ± 0.2</td>
<td>(12)</td>
</tr>
<tr>
<td>+ Total DNase†</td>
<td>1.9 ± 0.2</td>
<td>(9)</td>
</tr>
<tr>
<td>Tubulin preincubation†</td>
<td>3.3 ± 0.5</td>
<td>(11)</td>
</tr>
<tr>
<td>Tubulin control†</td>
<td>1.9 ± 0.4</td>
<td>(8)</td>
</tr>
</tbody>
</table>

- *Details of PME and PESS and reactivation buffer given in Materials and methods.
- †DNase treatments were at pH 7.5 for 2–4 min prior to reactivation: partial = 50–150 units ml⁻¹, total = 300–600 units ml⁻¹ until all detectable chromatin is digested.
- ‡Incubation in 2.2 mg ml⁻¹ tubulin under polymerizing conditions for 3–4 min prior to reactivation: Tubulin control incubated in media lacking only the tubulin.
### Table 3. Comparison of the initial length of the zone of microtubule overlap with the total increment of spindle elongation

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Overlap length (±SEM)</th>
<th>Total elongation (±SEM)</th>
<th>Difference (±SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PME</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 7.0</td>
<td>2.2±0.09</td>
<td>1.9±0.17</td>
<td>0.3</td>
</tr>
<tr>
<td>DNase, pH 7.0</td>
<td>1.9±0.21</td>
<td>2.1±0.08</td>
<td>-0.2</td>
</tr>
<tr>
<td>pH 7.5</td>
<td>2.0±0.09</td>
<td>1.8±0.14</td>
<td>0.2</td>
</tr>
<tr>
<td>DNase, pH 7.5</td>
<td>2.0±0.13</td>
<td>1.8±0.1</td>
<td>0.2</td>
</tr>
<tr>
<td><strong>PESS</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 7.0</td>
<td>2.1±0.13</td>
<td>2.2±0.13</td>
<td>-0.2</td>
</tr>
<tr>
<td>DNase, pH 7.0</td>
<td>1.9±0.13</td>
<td>1.9±0.17</td>
<td>0.0</td>
</tr>
<tr>
<td>Tubulin pretreatment Initial</td>
<td>1.9±0.08</td>
<td>3.5±0.31</td>
<td>-1.6</td>
</tr>
<tr>
<td>Final</td>
<td>3.4±0.21</td>
<td>6.1±0.7</td>
<td>-4.2</td>
</tr>
<tr>
<td>Tubulin+0.1 mM ATP†</td>
<td>1.9±0.09</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Treatments as given for Table 2, except final item.
†Data pooled for experiments with 1.1. and 2.2 mg ml⁻¹ tubulin, and for single and double perfusion protocols. Sample size=17.
‡Data include examples where elongation measurement was ended when spindle bent out of the focal plane.

When observed under phase-contrast, the rate of midzone shrinkage (−0.35±0.04 μm min⁻¹) is equal and opposite to the spindle elongation rate (0.4±0.03 μm min⁻¹; Fig. 2). However, the midzone often stops shrinking once its length falls below 1 μm, and remains visible for several minutes after elongation stops. In such examples, the length of the midzone at which it appears to be arrested was about at the limit of resolution for the optics used (Inoué, 1986) and so the observed midzonal density indicates only the existence but not the size of the contrast-producing material. Under polarized light, the midzone region of increased retardation vanishes as its length falls below 1 μm, with no evidence of any persistent material. The rate of midzone shrinkage in polarized light (−1.7±0.3 μm min⁻¹) is nearly twice the magnitude of the spindle elongation rate (0.9±0.09 μm min⁻¹); but this discrepancy probably occurs because the midzone loses its increased form birefringence when the separation of microtubule ends of each half-spindle approaches one half-wavelength of the illumination rather than when the ends actually separate (Sato et al. 1975). The persistence of contrast in the midzone under phase-contrast observation suggests that some substance is present between half-spindles, which initially is localized to the zone of microtubule overlap and becomes compacted during sliding.

**Simultaneous tubulin and ATP addition**

During anaphase B in intact *S. turris* the total spindle length roughly doubles, which shows that considerable tubulin polymerization occurs during spindle elongation in vivo (McDonald et al. 1986). Therefore, we wished to examine the kinetics of reactivation when ATP and tubulin are present together. Masuda and Cande (1987) developed conditions under which tubulin purified from bovine brain would incorporate into the diatom spindle; we have adapted these conditions for on-line observations. Preliminary results showed that reducing the ATP level from 1 to 0.1 mM produced rates of spindle elongation that were comparable to the rate of tubulin incorporation. With 0.1 mM ATP and 1.2 mg ml⁻¹ tubulin, spindles often doubled in length (Figs 4 and 5) and examples where spindle length more than doubled were found. The kinetics of elongation of these spindles are linear, as far as could be accurately determined (Fig. 6).

The average total elongation of spindles treated with both ATP and tubulin is 6 μm (Table 3), which underestimates the true average because in most instances one of the spindle poles is eventually driven out of focus during elongation. This large elongation increment is considerably greater than the amount of microtubule overlap initially present (Table 3). Our observations here (as well as those of Masuda and Cande, 1987; and Wordeman et al. 1989) are consistent with tubulin adding at the microtubule plus ends at the zone of overlap. First, no zone of altered contrast was apparent at the polar third of the half-spindles; one would expect a zone of lessened retardation in that area unless every microtubule were extended. Second, measurable elongation stops as soon as the half-spindles become completely separated; this is

![Fig. 4. Video micrographs with polarized light of an isolated spindle initially (A), at 6 min (B), and 10 min 40 s (C) after treatment with 0.1 mM ATP and 1.1 mg ml⁻¹ tubulin under polymerizing conditions. Note the total amount of elongation is approximately equal to the initial spindle length.](image-url)

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hard to explain if tubulin addition were occurring at the poles. We conclude, therefore, that the large increments of spindle elongation are caused by concomitant sliding apart of the half-spindles and tubulin addition in the zone of overlap.

After 4 μm of sliding, the microtubule overlap comprises predominantly brain microtubules. Nevertheless, constant elongation rates were unambiguously measured for as much as 6 or 8 μm of total elongation. Therefore the force-transducing system for anaphase B functions at the same rate for both diatom and brain microtubule substrates. Additionally, a consequence of observing increments of elongation that are greater than the entire initial spindle length is that the force transducer cannot be fixed on the diatom microtubules, but must be able to remain located between overlapping microtubules during spindle elongation.

**Tubulin polymerization prior to spindle elongation**

Masuda and Cande (1987) studied the rate of spindle elongation as a function of tubulin concentration and they obtained evidence that the elongation rate depended on the concentration of polymerizable tubulin. Moreover, Masuda et al. (1988) found that the effect of polymerizing tubulin on spindle elongation rate was retained when spindles were first incubated in tubulin for several minutes and only subsequently given ATP without tubulin to induce elongation. However, they inferred rates from observations on populations of fixed spindles measured at only two or three timepoints. We have performed tubulin preincubation experiments with direct observation to confirm the reported increase in elongation rate and to examine the kinetics of spindle elongation in the presence of prior tubulin polymerization.

When isolated spindles are incubated in tubulin (2.2 mg ml⁻¹) under polymerizing conditions, and viewed with polarized light, the midzone between half-spindles grows longer (Fig. 7). The rate of growth of the midzone depends on tubulin concentration, ranging between 0.2 and 2.4 μm min⁻¹ but usually less than 1 μm min⁻¹ (Fig. 8). Spindles were incubated in tubulin solutions for roughly 4 min, by which time the average midzone length is 3.4 μm (Table 3). The tubulin solution was then washed out with reactivation solution lacking ATP and then 1 mM ATP was perfused in. Elongation of such spindles is linear (Fig. 8), and the total increment of elongation equals the final, not the initial, length of the spindle midzone (Table 3). These data suggest that the exogenous tubulin adds on to the ends of the diatom microtubules, thus increasing in parallel the extent of the overlap zone and of spindle elongation. The average rate of elongation for tubulin-pretreated spindles is faster than those treated with zero tubulin (Table 2). This confirms the results of Masuda et al. (1988) that tubulin polymerization raises the
Fig. 7. Video micrographs, with polarized light optics, of an isolated spindle initially (A), after 2 min in 1.6 mg ml⁻¹ tubulin (B), and after 2.5 min in 1 mM ATP (C). A', B'. A nine pixel-wide line scan, parallel to the long axis of the spindle, showing grey levels of the spindle shown in A, B. Note that zone of overlap increases in length between A' and B' but that the minimum grey level does not decrease appreciably. Bar, 5 μm.

output of the force-transducing system, but does not act to generate force itself.

The site of incorporation of exogenous tubulin
When the site of incorporation into the spindle of biotinylated tubulin was studied with fluorescence microscopy of fixed material (Masuda and Candè, 1987; Masuda et al. 1988), biotinylated tubulin added in regions flanking the overlap zone and at the spindle poles. In on-line observations here, when spindles were incubated in underivatized tubulin, the length of isolated spindles viewed in polarized light does not grow (Fig. 8). Addition at the pole is therefore probably in the form of divergent, aster-like microtubules that are not an integral part of the central spindle. In the overlap zone, tubulin could become incorporated either by extending diatom microtubules (i.e. making heteropolymers), or by forming short microtubule fragments. Only a few fragments were detected in a serial-section analysis of a spindle incubated in tubulin without ATP (Masuda et al. 1988). We have tested for fragment formation by measuring the grey level of the polarized light image of the spindle during tubulin incubation. Production of microtubule fragments would be expected to decrease the grey level throughout the midzone (i.e. to cause a greater retardation). Inspection of the density scans in Fig. 7 suggests that the intensity minimum in the middle of the spindle spreads toward the poles during tubulin incorporation but does not decrease in value. This impression is confirmed by measuring the average grey level, at the center of the overlap zone and at a region completely outside the overlap zone, which remain nearly constant during incubation in tubulin (Fig. 9). Therefore, the evidence here from direct observation is consistent with that previously obtained with fluorescence and electron microscopy (Masuda et al. 1988); exogenous bovine brain tubulin is incorporated mainly at the ends of the microtubules in the midzone, thus producing heteropolymers.

Examination of Fig. 9 does show a slight increase in apparent retardation, in both of the measured sites. Small retardation increases were consistently observed, and amounted to a mean increase of approximately 1 grey level per minute (10 examples, s.e.m. = 0.3). This may represent a structural change in the spindle happening slowly during incubation and related to the observed promotion of spindle elongation rate by tubulin incorporation (Table 2), or alternatively may reflect the formation of a small number of microtubule fragments throughout the spindle.

An unexpected finding of the grey level measurements was that after ATP addition the retardation of the half-spindle remained constant during sliding (Fig. 9) and for several minutes after sliding had stopped and the two half-spindles were separated by a gap (not shown). Because the spindles often rotate or splay apart during sliding, measurements could not always be made, but data were
the zone of increased retardation. Retardation increases the rate-limiting step in the force-transduction basis of a biochemical or structural alteration that interacts with tubulin, are most simply explained on the transducing ATPase that functions at steady state. Palazzo, 1988). The steady rate of spindle elongation in the kinetics were not extensively studied (Rebhun and . . . .

Changes in rate, such as those induced by pH or preincubation with a factor that causes a change in the structure of the spindle, can be explained either because the site on the microtubule substrate is the rate-limiting step for spindle elongation. If rates of association and disassociation between motor and microtubule are not rate-limiting then it is possible that release of hydrolysed nucleotide products from the motor is rate-limiting. Product-release is thought to be rate-limiting for dynein (Johnson, 1985).

Furthermore, even if the site on the microtubule lattice recognized by the crossbridge protein has diverged, the affinity of the diatom motor for bovine microtubules is nevertheless sufficiently great to result in the noticeable slowing of the entire cycle, i.e. of elongation rate. The

Discussion

Characterization of the force-transducing system responsible for mitotic spindle elongation

The principal finding of this study is that the rate of mitotic spindle elongation in vitro is constant, throughout the time course of elongation, over a wide range of rates and conditions. A linear rate of spindle elongation has also been reported for isolated sea-urchin spindles, although the kinetics were not extensively studied (Rebhun and Palazzo, 1988). The steady rate of spindle elongation in vitro is most simply explained by the action of a force-transducing ATPase that functions at steady state. Changes in rate, such as those induced by pH or preincubation with tubulin, are most simply explained on the basis of a biochemical or structural alteration that changes the rate-limiting step in the force-transduction cycle. Other models in which force for spindle elongation is provided by the release of energy, stored in an elastic matrix stretched across the overlap zone (McIntosh, 1981) or in between microtubules (Snyder, 1988), predict a spindle elongation rate that would continuously decline with the extent of elongation. This prediction is not met by the data here. We suggest that the mechanochemical ATPase for spindle elongation follows the cycling-crossbridge model, which has been successfully applied to dynein and myosin (Johnson, 1985) as well as to other enzymes of intracellular motility (e.g. see Scholey et al. 1989).

If the motor molecules are spread throughout the zone of microtubule overlap, then as the extent of overlap between half-spindles lessens the load on each motor enzyme increases. The constant elongation rate means that the load on the motor enzymes is not rate-limiting. Evidence from vertebrate tissue culture cells to show that spindle elongation is not load-limited in vivo has also been obtained (Taylor, 1965). Because the isolated spindles studied here elongate at a well-defined velocity, and are surrounded by a medium of known viscosity (i.e. water), the force to overcome viscous drag is readily calculated as the product of velocity (2 μm min^-1) times viscosity (10^-3 Pa s) times a shape factor (50 μm) (Nicklas, 1965; Purcell, 1977). The force thus calculated is of the order of 10^-15 N. Calculating the work done by this force in 2 μm of elongation gives 3.2 × 10^-14 erg, or about 6% of the energy from hydrolysis of the terminal phosphate bond of a single ATP. The load due to viscosity is therefore trivial.

Given the hydrolysis during spindle elongation of presumably more than 0.06 of a molecule of ATP, why is the rate of spindle elongation so slow? Measurements in echinoderm eggs of the force produced by the spindle during elongation suggest that the spindle can exert six to seven orders of magnitude more force than that calculated above to overcome viscous drag (Hiramoto and Nakano, 1988; for chromosome to pole movement, see Nicklas, 1983). Because isolated spindles that are surrounded by a cloud of chromatin can elongate at similar rates to those in which the chromatin has been removed (Table 2), it is unlikely that spindle binding to the glass substratum imposes a velocity-limiting load. This conclusion is strengthened by observations of DNase-treated spindles undergoing Brownian (angular) motion around an anchored pole while elongating at typical rates. Resistance to force generation would arise if the rate-limiting step of the motor occurs while the ATPase is bound to the microtubule substrate. At any time, most of the bound ATPase molecules would act as rigid crossbridges and would resist the force exerted by the few ATPase molecules then in the power-stroke phase of the cycle (Huxley, 1957).

The rate of spindle elongation was also constant for sliding in exogenous tubulin despite the transition from an interaction between diatom motors and microtubules to an interaction between diatom motors and mammalian microtubules (Figs 6 and 8). The constant reaction rate can be explained either because the site on the microtubule lattice recognized by the motor protein is completely conserved between diatoms and cows or because neither binding nor dissociation of the crossbridge and microtubule substrate is the rate-limiting step for elongation. If rates of association and dissociation between motor and microtubule are not rate-limiting then it is possible that release of hydrolysed nucleotide products from the motor is rate-limiting. Product-release is thought to be rate-limiting for dynein (Johnson, 1985).

Furthermore, even if the site on the microtubule lattice recognized by the crossbridge protein has diverged, the affinity of the diatom motor for bovine microtubules is nevertheless sufficiently great to result in the noticeable slowing of the entire cycle, i.e. of elongation rate. The

Fig. 9. Grey level versus time measurements for two regions on an isolated spindle, treated with a protocol similar to that for Figs 7 and 8, except that a second ATP perfusion was required to initiate spindle elongation. (□) Data for a region in the center of the zone of increased retardation (i.e. the spindle midzone); ( △) data for a region on the half-spindle fully outside the zone of increased retardation. Retardation increases downward on the ordinate. Note approximately constant grey level of the background adjacent to the spindle (background level near 55; 148 digitized grey levels from zero to 256). Just prior to time = 1 min, 1.1 mg ml^-1 tubulin was added under polymerizing conditions. At the first arrow, tubulin solution was washed out and then an ATP solution (0.1 mM) was immediately washed in, and a repeat ATP perfusion was given at the second arrow.

obtained in a number of favorable instances where appreciable change in spindle structure did not happen during sliding, both with and without tubulin pretreatment. A constant retardation of the half-spindle during sliding was confirmed. This indicates that significant numbers of microtubules were not lost from the half-spindles during sliding, although depolymerization at the edges of the gap that did not reach the measured site on the half-spindle cannot be excluded.
The effect of tubulin on the rate of spindle elongation

Masuda and Cande (1987) reported that the rate of spindle elongation is increased by simultaneous incubation of the spindle in tubulin and ATP as compared to that of spindles incubated in ATP alone. The reason for the promotion of elongation rate by tubulin is not understood, but several possibilities may now be excluded. The finding here of linear elongation rates with and without tubulin, and of no specific time lag before the onset of elongation, shows that the effect of tubulin cannot be explained by different kinetics with and without tubulin. The linear elongation rates also show that the promotion of elongation rate occurs initially, even before appreciable amounts of added tubulin can have entered the overlap zone. The promotion of elongation rate by tubulin also cannot be the result of simply an increase in the size of the zone of microtubule overlap, because the size of the overlap zone never correlated with elongation rate (Figs 6 and 8). Finally, the promotion of spindle elongation rate by tubulin cannot be explained by polymerization, because the increase in rate occurs even when the tubulin is removed before ATP addition (Table 2; Masuda et al. 1988); and because the concentration of polymerizable tubulin probably falls continuously from the start of perfusion, as indicated by the rapid filling of the perfusion chamber with microtubules, without deceleration in spindle elongation rate, even after many minutes in the presence of tubulin (Fig. 6). The ability of tubulin addition to increase the efficacy of the force-transduction cycle remains unexplained.

Further indication of a link between tubulin addition and the force-generating system for spindle elongation is seen in the tendency for the overlap zone to be swept out of the overlap zone and elongation would be expected to stop after a distance of roughly twice the overlap zone length (i.e. 4 \mu m) had been reached. Masuda and Cande (1987) drew similar conclusions on the basis of indirect observations. The phase-dense material seen to remain between half-spindles here, and previously (Baskin and Cande, 1988), and the thio phosphorylated antigens localized to the spindle midzone in several species (Kuriyama, 1989; Wordsen et al. 1989) indicate the existence in the spindle midzone of a matrix, of which the force-transducing ATPase is probably a component.

An additional characteristic of the anaphase B molecular motor observed here is its ability to promote sliding between microtubules from each half-spindle that are not 180 degrees apart, i.e. are not strictly antiparallel (Fig. 4). In some examples, after ATP perfusion, elongation occurs only in a small peripheral bundle of microtubules: elongation drives the bundle laterally away from the inactive central spindle while the angle between the sliding microtubules departs more and more from 180 degrees. In anaphase of Haemanthus endosperm cells, lateral outward movements of small bundles of microtubules have been seen (Lambert and Bajer, 1972); in micrographs of anaphase in various organisms, microtubules from each spindle pole are seen to overlap at angles other than 180 degrees (e.g. see Roth et al. 1966). The extensive order of the diatom central spindle is often contrasted with the less well-ordered spindles of many other types of organism (Pickett-Heaps and Tippit, 1978), but this extensive order is probably not a requirement for the force-transducing system studied here. The unit of interaction for anaphase B may be no more than two approximately antiparallel microtubules linked by the force-transducing enzyme and any required matrix.

The effect of tubulin on the rate of spindle elongation

Masuda and Cande (1987) reported that the rate of spindle elongation is increased by simultaneous incubation of the spindle in tubulin and ATP as compared to that of spindles incubated in ATP alone. The reason for the promotion of elongation rate by tubulin is not understood, but several possibilities may now be excluded. The finding here of linear elongation rates with and without tubulin, and of no specific time lag before the onset of elongation, shows that...
spindles after they had separated. In the isolated spindles, we never observed bulk depolymerization of half-spindles within roughly 5 min after the completion of sliding. Cande and McDonald (1986), however, have shown evidence for an appreciable (but not total) loss of microtubules from the half-spindles at longer times after sliding (5–10 min). An aspect of the isolation procedure used here could stabilize the spindle microtubule plus ends against ATP-induced depolymerization, or depolymerization in the cell could be promoted by a soluble factor that is lost during isolation.

Conclusion

We have shown that the elongation of isolated diatom mitotic spindles is linear, over a range of rates, and despite the presence of heterologous microtubules as substrates. This behavior suggests that the motility of anaphase B is driven by a mechanochemical ATPase having a cycle of steps in which energy from ATP hydrolysis is converted to force-generation for sliding. The ATPase must remain in the work of microtubule sliding. Neither polymerization nor depolymerization of tubulin is likely to contribute to part of a phase-dense matrix that is observed to have such force-generation for sliding. The ATPase must remain in the midzone of the spindle during sliding, and it may be part of a phase-dense matrix that is observed to have such behavior. Finally, the ability of the force-transducing system from the diatom to function normally with brain tubulin as its substrate suggests that a force-transducing enzyme, homologous to that of the diatom, located in the zone of overlap between half-spindles, powers spindle elongation in many kinds of organisms.

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