Modulation of Microtubule Stability by Kinetochores In Vitro

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Abstract. The interface between kinetochores and microtubules in the mitotic spindle is known to be dynamic. Kinetochore microtubules can both polymerize and depolymerize, and their dynamic behavior is intimately related to chromosome movement. In this paper we investigate the influence of kinetochores on the inherent dynamic behavior of microtubules using an in vitro assay. The dynamics of microtubule plus ends attached to kinetochores are compared to those of free plus ends in the same solution. We show that microtubules attached to kinetochores exhibit the full range of dynamic instability behavior, but at altered transition rates. Surprisingly, we find that kinetochores increase the rate at which microtubule ends transit from growing to shrinking. This result contradicts our previous findings (Mitchison, T. J., and M. W. Kirschner, 1985*b*) for technical reasons which are discussed. We suggest that catalysis of the growing to shrinking transition by kinetochores may account for selective depolymerization of kinetochore microtubules during anaphase in vivo. We also investigate the effects of a nonhydrolyzable ATP analogue on kinetochore microtubule dynamics. We find that 5′ adenylylimido diphosphate induces a rigor state at the kinetochore–microtubule interface, which prevents depolymerization of the microtubule.

MICROTUBULES exhibit dynamic instability in vitro and in vivo: individual microtubules in a steady-state population exist in growing and shrinking states (Horio and Hotani, 1986; Mitchison and Kirschner, 1984), and transit between them at a rate that varies under different conditions (Walker et al., 1988). In this paper we will continue the convention of calling the first-order rates at which microtubules go from growing to shrinking the catastrophe rate, and from shrinking to growing the rescue rate. Dynamic instability together with control of catastrophe and rescue rates is thought to regulate microtubule distribution and function in the cell (Kirschner and Mitchison, 1986).

Mitotic spindles contain at least two different classes of microtubules: astral microtubules, which have free plus ends, and kinetochore microtubules, whose plus ends attach to chromosomes at their kinetochores (Rieder, 1982; Roos, 1973). These two classes exhibit very different dynamic behavior in the course of mitosis. During metaphase, astral microtubules turn over more rapidly than kinetochore microtubules (Mitchison et al., 1986; Wadsworth and Salmon, 1986). During anaphase, astral microtubules mostly grow while kinetochore microtubules progressively shrink (Bajer and Mole-Bajer, 1972; Vandre et al., 1984). Kinetochore microtubule dynamics are closely coupled to chromosome movement, so that movement of chromosomes on the spindle axis is almost always coupled to length changes of kinetochore microtubules (Bajer, 1982; Inoue, 1976; Ostergren, 1951; Roos, 1973; Salmon, 1976); but also see Rieder (1990) for an important exception. Most of these length changes are thought to occur by addition or loss of subunits at microtubule ends attached to kinetochores; microtubules polymerize at kinetochores during prometaphase and metaphase when chromosomes are moving away from poles or maintaining constant distance (Mitchison, 1989; Mitchison et al., 1986). They depolymerize at kinetochores during anaphase when chromosomes are moving towards poles (Mitchison et al., 1986). Given these facts, we need to explain two aspects of kinetochore microtubule dynamics (*a*) how are they differentiated from astral microtubule dynamics; and (*b*) what determines whether microtubules polymerize or depolymerize at the kinetochore.

The dynamic behavior of kinetochore microtubules could be controlled in at least two ways: by binding of specific microtubule-associated proteins (Horio and Hotani, 1986; Job et al., 1987; Vallee et al., 1984), or directly by interaction with the kinetochore (Mitchison, 1989). The complexity of spindle organization and chemistry make it very difficult to distinguish factors affecting the dynamics of kinetochore microtubules in living cells. Recently, it has become possible to analyze this problem in vitro, by reconstituting the interaction between kinetochores and microtubules in the absence of other cellular components (Koshland et al., 1988; Mitchison and Kirschner, 1985*a*). The kinetochores of isolated CHO chromosomes can acquire attached microtubules in two ways: by nucleation (McGill and Brinkley, 1975; Mitchison and Kirschner, 1985*b*; Telzer et al., 1975) or capture of preformed microtubules from solution. Both nucleation and capture result in attached microtubules with both plus and minus ends distal to the kinetochore (Mitchison and Kirschner, 1985*a*; Mitchison and Kirschner, 1985*b*). In both cases the proportion of microtubules that at-

tach at their ends relative to those making lateral connections has not been established. After nucleation, the microtubule end at the kinetochore–microtubule interface has not been observed to show any polymerization and depolymerization (Mitchison and Kirschner, 1985*a*), but the microtubule end resulting from capture has been shown to be highly dynamic. Two reactions after capture have been studied in detail as follows.

(*a*) Polymerization at kinetochores. When labeled microtubules were captured by kinetochores and unlabeled tubulin was then added at concentrations sufficient to promote polymerization (10–30 μM), the labeled microtubules polymerized from both ends. When ATP was subsequently added to the mixture, the kinetochores moved towards the plus ends of these attached, polymerizing microtubules. With tubulin and ATP simultaneously present, polymerization and movement were concomitant, and the labeled microtubules were extruded away from the kinetochores with their minus ends distal. When tubulin was added first without ATP, polymerization occurred without movement. When ATP was added subsequently, the kinetochores moved towards plus ends in the absence of further polymerization. These results were interpreted to indicate the existence of a plus end–directed ATPase in the kinetochore (Mitchison and Kirschner, 1985*a*). Because the ATP-dependent movement was unidirectional, captured seeds elongated at the kinetochore always have their plus ends proximal.

(*b*) Depolymerization at kinetochores. The development of cross-linked labeled microtubules allowed the observation of microtubule depolymerization at kinetochores. These labeled microtubules were initially elongated from their plus ends while free in solution, using unlabeled tubulin, and then captured by kinetochores. When the resulting complexes were diluted, the unlabeled segment depolymerized while remaining attached to the kinetochore (Koshland et al., 1988). This depolymerization with concomitant movement of the kinetochore towards the minus end of the microtubule did not require ATP, and proceeded even when nucleotide triphosphates were depleted.

These studies have left many questions unanswered, including: can microtubules switch from growing to shrinking while remaining attached to kinetochores; what is the quantitative effect of kinetochore binding on microtubule stability; and does the in vitro reaction produce force at levels known to exist in vivo? The first two of these questions are addressed in this paper. We use the in vitro assay to probe how the kinetochores of isolated chromosomes affect microtubule dynamics, by comparing the behavior of kinetochore-attached and free microtubule ends. The implication of these results for chromosome movement and kinetochore structure are discussed.

Materials and Methods

The experimental protocols used are based on methods previously published (Koshland et al., 1988; Mitchison and Kirschner, 1985*a*). Tubulin refers to phosphocellulose-purified bovine brain tubulin. All incubations were at 37°C.

Labeled Microtubule Seed Construction

Stable biotin-labeled microtubules were made by polymerizing biotin tubulin (25 μM) in BRB80[1] (80 mM Pipes, 1 mM $MgCl_2$, 1 mM EGTA, pH 6.8, with KOH) in the presence of the nonhydrolyzable analogue guanylyl-αβ-methylenediphosphonate (GMPCPP) (no commercial source available) for 15 min. GMPCPP microtubules show no length changes when extensively diluted in BRB80 without tubulin for up to an hour at 37°C, and many persist for 12 h. Detailed procedures for stable seeds have been described in Hyman et al. (1990). For the experiment in Fig. 5, unstable, biotin-labeled microtubules were made by polymerizing 25 μM tubulin and 5 μM biotin tubulin in BRB80 containing 20% glycerol, 3 mM $MgCl_2$, 1 mM GTP for 15 min. They were sheared through a 27-gauge needle and left for 5 min before use.

Capture Reaction

CHO chromosomes (Mitchison and Kirschner, 1985*a*) were diluted 1:5 in BRB80 containing 0.1% beta-mercaptoethanol (BME), 2 μM tubulin, 2 mM GTPγS, and a 40-fold dilution of GMPCPP microtubules and incubated for 10 min. For Fig. 5, the conditions were the same except that the labeled microtubules were diluted 10-fold, and the reaction included 2 μM biotin tubulin, 10 μM unlabeled tubulin, and 1 mM GTP to prevent depolymerization of the labeled microtubules during the capture reaction.

Plus End–directed Movement

After the capture reaction had progressed for 10 min, it was diluted 20-fold into BRB80 containing tubulin at concentrations as detailed in the figure legends, and 1 mM GTP, 1 mM ATP, 2 mM $MgCl_2$, and 0.1% beta-mercaptoethanol. The dilution was performed to prevent capture of new microtubules during subsequent incubations. Control experiments in which labeled microtubules were diluted before chromosome addition demonstrated that no capture occurred after a 20-fold dilution.

Minus End–directed Movement

In the experiments of Figs. 1, 2, 6, and 7, the complexes generated by plus end–directed movement were diluted a further 20-fold into 12% glycerol in BRB80 in the absence of further GTP or ATP. In the experiment in Fig. 3 dilution was in BRB80 plus 10 μM tubulin plus 1 mM GTP. To avoid shearing, free microtubules were analyzed from an aliquot diluted at an earlier time than the kinetochore microtubules.

Fixation and Immunofluorescence

Reactions were fixed by dilution of aliquots into 5–10 mM ethyleneglycol-bis-succinimidylsuccinate in BRB80 + 10% DMSO. Complexes and free microtubules were sedimented separately onto coverslips and stained as described (Mitchison and Kirschner, 1985*a*). For immunofluorescence, all the microtubules were visualized in the fluorescein channel and the biotin microtubule in the rhodamine channel as described in Mitchison and Kirschner (1985*a*). In addition, unlabeled microtubules were visualized faintly in the rhodamine channel by adding a low concentration of rhodamine secondary antibody to the fluorescein secondary antibody. This allowed length measurements from a single image in the rhodamine channel without resort to registration between fluorescein and rhodamine images. Rhodamine images were recorded using a camera (model SIT; Cohu Inc., San Diego, CA) onto an optical memory disc recorder (Panasonic, Japan), and digitized on a video monitor using Java software (Jandel Inc., Corte Madeira, CA). Operator bias was prevented as follows: a chromosome was chosen by Hoechst staining, and the microtubules were viewed in the fluorescein channel (where the labeled segments could not be seen). If the microtubules appeared unbroken and emanated from a single focus at the kinetochore, the complex was recorded in the rhodamine channel and digitized.

Statistical Test of Microtubule Shortening

The null hypothesis was that apparent A segment shortening after dilution in Fig. 2 was in fact due only to selective loss of long microtubules from the predilution population. To extrapolate the length distribution at the time of dilution, we took the length distribution of A segments at 10 min in Fig. 2 *c*, and added 1.0 μm to the mean length. This reflects the growth rate of

1. *Abbreviations used in this paper*: AMPPNP, 5′ adenylylimido diphosphate; BRB 80, 80 mM Pipes, 1 mM $MgCl_2$, 1 mM EGTA, pH 6.8, with KOH; GMPCPP, guanylyl-αβ-methylenediphosphonate; NEM, *N*-ethylmaleimide.

1 μm/min in this population. We then derived a hypothetical length distribution by removing the longest microtubules until the population was reduced in number by 32%. This was the fractional loss of microtubules in the population fixed at 12 min. We compared this hypothetical distribution to the observed distribution at 12 min. The mean of the hypothetical population was 8.2, SD = 2.4, compared with mean 6.2, SD = 3.1 for the real population. These populations are significantly different using a two-tailed t test ($p < 0.002$). Even without adding the extra micron to the 10-min distribution, the distribution at 12 min is very unlikely to have arisen by selective loss of long microtubules ($p < 0.1$). We reject the null hypothesis, and conclude that the short microtubules in the diluted population must have arisen by actual shrinkage of microtubules from the predilution population.

Estimation of Transition Rates for Fig. 4

Stable, labeled seeds were incubated with chromosomes for 10 min, and the complexes were diluted into buffer containing 25 μM tubulin, 1 mM GTP, 1 mM ATP, and 15 μM *N*-ethylmaleimide (NEM)-tubulin. The NEM-tubulin does not effect plus ends' polymerization or movement of seeds away from kinetochores, but prevents polymerization onto free minus ends. The preparation and properties of the reagent are described in Hyman et al. (1990). After 10 min the mixture was diluted 20-fold to give the final tubulin concentration indicated, in a solution containing one part biotin-labeled tubulin to four parts unlabeled tubulin. The biotin tubulin was used to detect growing plus ends on free microtubules. Complexes were fixed 1 min after the final dilution and analyzed for kinetochore and free microtubules.

To estimate the catastrophe rate for kinetochore microtubules, we counted the average number of microtubules displaying a clear A segment per chromosome for 60–120 chromosomes fixed just before, and 1 min after dilution. The ratio of these numbers was considered the fraction of microtubules that had grown continuously after dilution. This fraction was used to estimate the transition rate, assuming that the loss of growing microtubules was a first-order process, using the formula: catastrophe rate = $\ln(N_0/N_t)/t$. Most complexes with a clear A segment represent microtubules still growing at the kinetochore, although a small fraction will be those caught in the act of shrinking. Inclusion of such shrinking microtubules leads to potential overestimation of the number of microtubules still growing. A second source of possible overestimation of the growing fraction arises if labeled segments shrink back to the kinetochore, then reverse direction and start growing again; that is, rescue of kinetochore microtubules. We did not try to correct for these factors leading to potential overestimation for the fraction of kinetochore microtubules still growing, and thus our estimate of catastrophe rates for kinetochore microtubules err if anything on the side of underestimates.

To estimate the catastrophe rate for free microtubules, the number of microtubules still growing 1 min after dilution was determined by visualizing the biotin segment. We scored three classes of microtubules. (*a*) Growing: these had a bright biotin segment (the initial seed) on one end, an unlabeled middle segment of the length expected if no shrinkage had occurred, and a short biotin segment on the other end which had polymerized after dilution. (*b*) Rescued: these were as in *a* except that the internal unlabeled segment was short enough that it must have depolymerized and then rescued. (*c*) Depolymerized: these were biotin labeled throughout. The population in fraction *b* was ascertained by comparing length histograms for the unlabeled segment before and after dilution. Microtubules whose unlabeled segment were shorter than the 95% confidence limit of the predilution histogram were counted as microtubules that had rescued. The number in population *b* was always <7% of the total, and thus represented a small correction to the data. The fraction of free microtubules that had continued to grow after dilution was scored as 1/1+2+3. It was used to estimate the transition rate assuming first-order loss as above.

Results

Assaying Microtubule Dynamics at Kinetochores

To investigate the dynamics of microtubule plus ends attached to kinetochores we needed to make complexes between microtubules and kinetochore with several properties. (*a*) The microtubules must be attached to kinetochores at their plus ends, and these ends must be capable of polymerizing and depolymerizing. (*b*) The minus ends of the microtubules must be blocked to depolymerization to allow assay of dynamics at the plus ends without disappearance of the microtubules. (*c*) An internal segment of the microtubules must be marked so that movement of the lattice with respect to the kinetochores can be followed. Previously, such complexes were made by first polymerizing from cross-linked, labeled microtubules, and then capturing the resulting long microtubules (Koshland et al., 1988). We found that cross-linked seeds tended to stick nonspecifically to kinetochores, and that long microtubules were captured inefficiently. Therefore, we set up an improved substrate. We made stable, biotin-labeled microtubule seeds by polymerization in the presence of a nonhydrolyzable GTP analogue (GMPCPP). These were captured efficiently by kinetochores. We then elongated the seeds in the presence of unlabeled tubulin and ATP. Under these conditions, the seed moves away from the kinetochore with the plus end always proximal (Mitchison and Kirschner, 1985*a*). Complexes made in this way are shown in Fig. 1. Length A is the segment of unlabeled microtubule polymerized by incorporation onto the plus end at the kinetochore, B is the stable, biotin-labeled microtubule, and C is the unlabeled segment polymerized onto the minus end of the labeled microtubule. When referring to the presence or absence of A segments in the text, we mean that a labeled B segment is visible connected to the kinetochore via an unlabeled A segment. We shall refer to these segments by their letter in the rest of the text. The labile A segment added at the kinetochore fulfills requirement *a* above. This method results in complexes with only plus ends at the kinetochore because of the directionality of the kinetochore ATPase as discussed in the introduction. The stable B segment fulfills requirement *b* and *c*. We were now in a position to assay the dynamic properties of the kinetochore–microtubule plus end interface. Fig. 1 also shows microtubules without labeled internal B segments that were nucleated at the kinetochore, and seeds that did not move away from the kinetochore during the assay. These could not provide information about dynamics at the kinetochore and they were disregarded.

Microtubules Can Transit from Growing to Shrinking while Attached to Kinetochores

We first wanted to know whether microtubules could remain attached to kinetochores when their ends switched between growing and shrinking phases. Stable, labeled seeds were incubated with CHO chromosomes for 10 min so that a small proportion of the labeled seeds were captured. These complexes were then diluted into 25 μM unlabeled tubulin, GTP, and ATP at time zero (Fig. 2) creating complexes as shown in Fig. 1. We measured the length at three time points and plotted this rate as a function of time. During this phase of the reaction the average distance between seeds and kinetochores increases linearly with time (Fig. 2 *a*). The number of A segments per kinetochore decreases slowly due to microtubule detachment (Fig. 2 *b*). This shows that kinetochores remain attached to microtubule plus ends as they polymerize, with a slow rate of detachment.

To test whether kinetochores remain attached when microtubules transit from growing to shrinking, we diluted these complexes undergoing plus end–directed movement to 1.25 μM tubulin in buffer containing 12% glycerol, at 11

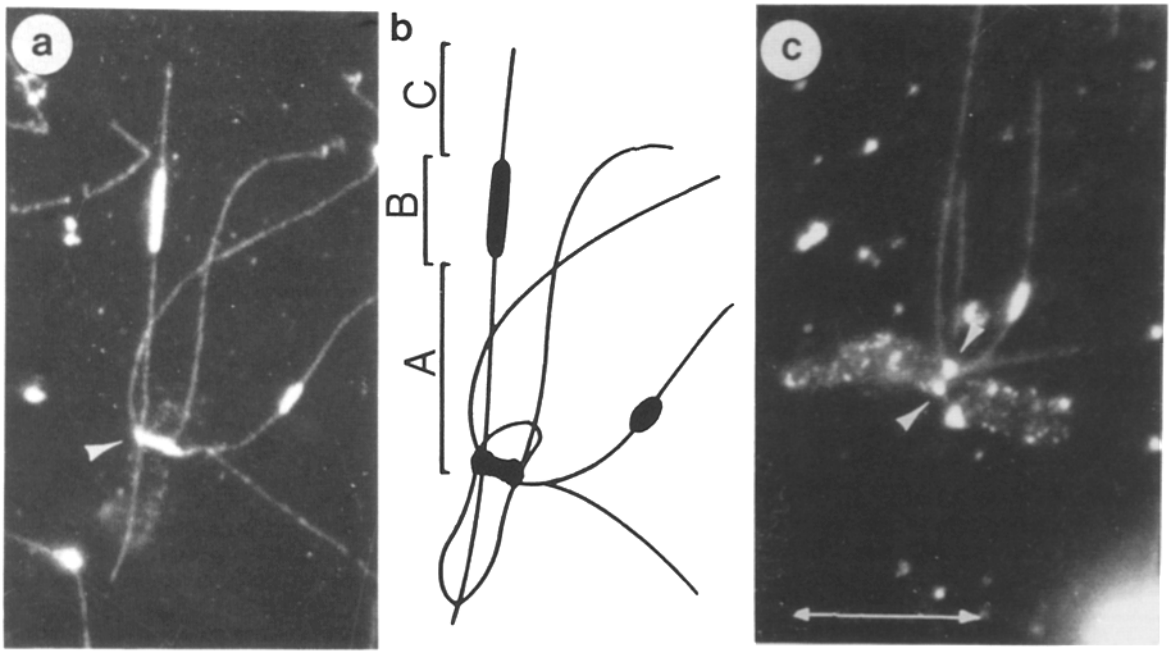

Figure 1. Kinetochore-microtubule complexes fixed during plus end-directed movement, and visualized by immunofluorescence. Stable biotin-labeled seeds were captured by mitotic CHO chromosomes, and then the complexes were diluted into buffer containing 25 μM tubulin, 1 mM GTP, 1 mM ATP, and fixed 10 min later. They were then sedimented onto coverslips through glycerol cushions, postfixed in methanol and stained sequentially with (*1*) rabbit antibiotin followed by rhodamine goat antirabbit to visualize stable seeds; (*2*) mouse antitubulin followed by fluorescein goat antimouse containing rhodamine antimouse at 1/20th the normal concentration to visualize total microtubules; and (*3*) hoecshst 33258 to visualize chromosomes. *a* and *c* show complexes visualized in the rhodamine channel. The stable seeds are very bright, and the unlabeled microtubule segments can be seen dimly by virtue of the low concentration of rhodamine in the antimouse secondary. The kinetochores (*arrows*) are visualized by virtue of their binding of tubulin. The outline of the chromosome can be seen faintly, especially in *c*. *b* shows a tracing of the complex in *a*. The initially captured, biotin-labeled microtubule is segment B. Segment B has elongated from both its minus end (*segment C*) and plus end (*segment A*) with unlabeled tubulin subunits. Bar, 10 μm.

min. At this concentration of tubulin, we expected that all microtubules would transit to shrinking. Glycerol was added to slow down microtubule depolymerization and thus spread out the time course of the reaction (Koshland et al., 1988). After dilution, the average length of A segments in populations of fixed complexes progressively decreased (Fig. 2 *a*). At the same time, the number of A segments per chromosome decreased at a rate higher than that during polymerization (Fig. 2 *b*). Examination of histograms (Fig. 1, *c–e*) showed that the decrease in average A was accompanied by a shifting of the length distribution to shorter lengths. A statistical test (see Materials and Methods) showed that the decrease in average length could not be due to selective loss of longer A lengths from the population. Rather, the decrease in average A length after dilution must reflect shortening of A segments. Since the B segment is completely stable under these conditions, this must be due to depolymerization of microtubule ends while they remained attached to kinetochores.

Minus end-directed movement of kinetochores induced by tubulin dilution has been observed previously, and is thought to be driven solely by the drive towards depolymerization of the microtubule lattice (Koshland et al., 1988). This is the first time that kinetochores have been observed to switch from plus end- to minus end-directed movement along the same microtubules.

Kinetochore Microtubules Are Less Stable than Free Microtubules

In the previous experiment, we had driven kinetochore microtubules first to grow and then to shrink by changing the tubulin concentration. Next, we wanted to compare the dynamic behavior of free and kinetochore microtubules at more physiological tubulin conditions, those at which free microtubule ends alternate between growing and shrinking states (Mitchison and Kirschner, 1984; Walker et al., 1988). We could compare free and kinetochore microtubules from the same reactions because the majority of labeled microtubule seeds in the capture incubation remain free in solution. When the capture reactions are diluted into tubulin solutions these free microtubules simply elongate off both ends. Their number concentration is too low for significant new capture to occur (Koshland et al., 1988; Mitchison and Kirschner, 1985*a*). These free microtubules from the same reactions can be studied separately from the chromosomes by extensive dilution and then sedimenting them onto coverslips at much higher *g* forces. Their plus ends can be distinguished from their minus ends due to their faster elongation rate.

To compare kinetochore and free microtubule dynamics we set up a reaction exactly as in Fig. 2, but diluting the complexes into 10 μM tubulin in buffer without glycerol. At this concentration of tubulin, microtubules were expected to

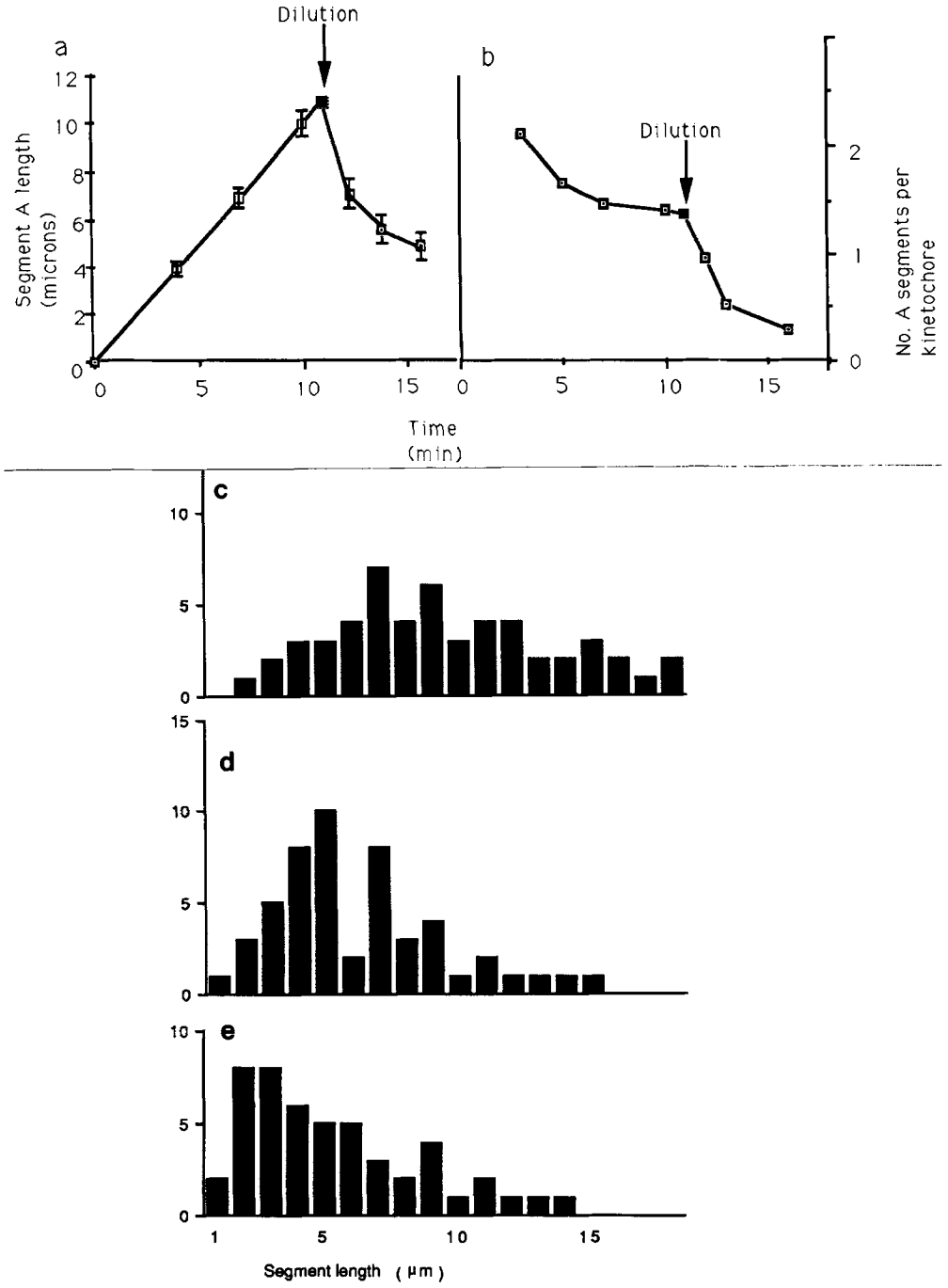

Figure 2. Microtubules plus ends can switch from growing to shrinking while remaining attached to kinetochores. Stable biotin-labeled seeds were captured by mitotic CHO chromosomes. At time 0 on the graph, chromosomes were diluted into buffer containing 25 μM tubulin, 1 mM GTP, 1 mM ATP to allow polymerization of the seeds and movement of kinetochores towards the plus end growing from this seed. At 11 min, the complexes were diluted 1:20 into buffer containing 12% glycerol (to slow microtubule depolymerization) and no nucleotides to give a final tubulin concentration of 1.25 μM. Aliquots were fixed at various time points and stained as in Fig. 1. The lengths of the A segments were determined by digitizing images like Fig. 1 *a* collected with an SIT video camera. (*a*) Average length of A segments before and after dilution. Error bars are the standard error, i.e., the standard deviation divided by the square root of the sample size. (*b*) Number of microtubules per kinetochore displaying A and B segments. (*c*) Length histogram of A segments at 10 min. (*d*) Length histogram of A segments at 12 min. (*e*) Length histogram of A segments at 16 min. Filled square is extrapolated time point. 40–80 A segments were measured per time point.

spend most time in a growth phase, but occasionally transit to shrinking (Mitchison and Kirschner, 1984; Walker et al., 1988). As expected, the plus ends of free microtubules continued to grow after dilution, but at a slower rate (Fig. 3). There is no depolymerization of the stable seeds under these conditions, and very few seeds did not have unlabeled segments growing from them.

To our surprise, the average length of kinetochore microtubule A segments decreased after dilution (Fig. 3), and after 30 s 80% of the A segment microtubules had disappeared (data not shown). We cannot accurately determine how much of this number loss was due to complete shrinkage, and how much to detachment, but since both processes are highly dependent on tubulin concentration (see below) they presumably both reflect catastrophe events at the attached microtubule end as discussed above. This means that kinetochore microtubule plus ends have a higher catastrophe rate than free plus ends at 10 μM tubulin.

Catastrophe Rates for Free and Kinetochore Microtubules

Under conditions of dynamic instability, microtubules transit from growing to shrinking, at a rate that depends upon the tubulin concentration (Mitchison and Kirschner, 1984; Walker et al., 1988). If the decrease in A segment length and

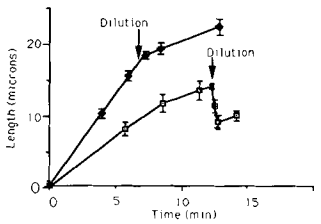

Figure 3. Kinetochore microtubules undergo catastrophe transitions more frequently than free microtubules at 10 μM tubulin. Stable, labeled seeds were captured by kinetochores. At time 0, plus end-directed movement was initiated at the kinetochore in 25 μm tubulin as described in Fig. 2. After 7 and 12 min, aliquots of the plus end-directed movement reaction were diluted 1:20 in buffer containing 10 μM tubulin, and 1 mM GTP but no ATP, in the absence of glycerol. Aliquots were fixed at various time points and processed as in Fig. 1, and digitized as in Fig. 2. The free microtubules were analyzed from the aliquot diluted at 7 min to avoid problems of shearing encountered when the microtubules grew more than 40 μm long. The plus end of free microtubules was defined as the longer of the two unlabeled segments growing from the labeled seed. (*Open squares*) A segments of kinetochore microtubules. (*Filled diamonds*) Unlabeled plus end segments of free microtubules. (*Closed square*) Extrapolated time point 50–90 microtubules/time point. Error bars are standard error of the mean, i.e., the standard deviation divided by the square root of the sample size.

A segment number after dilution into 10 μM tubulin was due to catastrophe transitions of kinetochore microtubules, we might expect that increasing the tubulin concentration would prevent this decrease. We decided to assay one of these parameters, the change in A segment number, after dilution into different tubulin concentrations.

Capture of labeled microtubules, and elongation of both captured and free ends was initiated as in the previous experiment, with one modification. We added NEM-modified tubulin to the incubation to confine all polymerization to plus ends (Huitorel and Kirschner, 1988; Hyman et al., 1990). This was not necessary to define kinetochore microtubule polarity, but it ensured that the unlabeled segments we analyzed on free microtubules were always plus ends, and therefore that we could directly compare kinetochore microtubule behavior with that of free microtubules. After 10 min in 25 μM tubulin plus ATP, the complexes were diluted to various test tubulin concentrations. The method for scoring growing plus ends required that these test tubulin concentrations contained biotin-labeled tubulin, but this was found not to affect polymerization rates (not shown). We then measured the fraction of kinetochore microtubules that had A segments 1 min after dilution, and also the fraction of free microtubules that had not undergone catastrophes (see Materials and Methods). Fig. 4 *a* shows the number of A segments remaining per kinetochore. After dilution into 30 μM tubulin there was no decrease in A segment number, indicating that loss at lower tubulin concentrations was not due to the physical manipulations involved in dilution. The extent

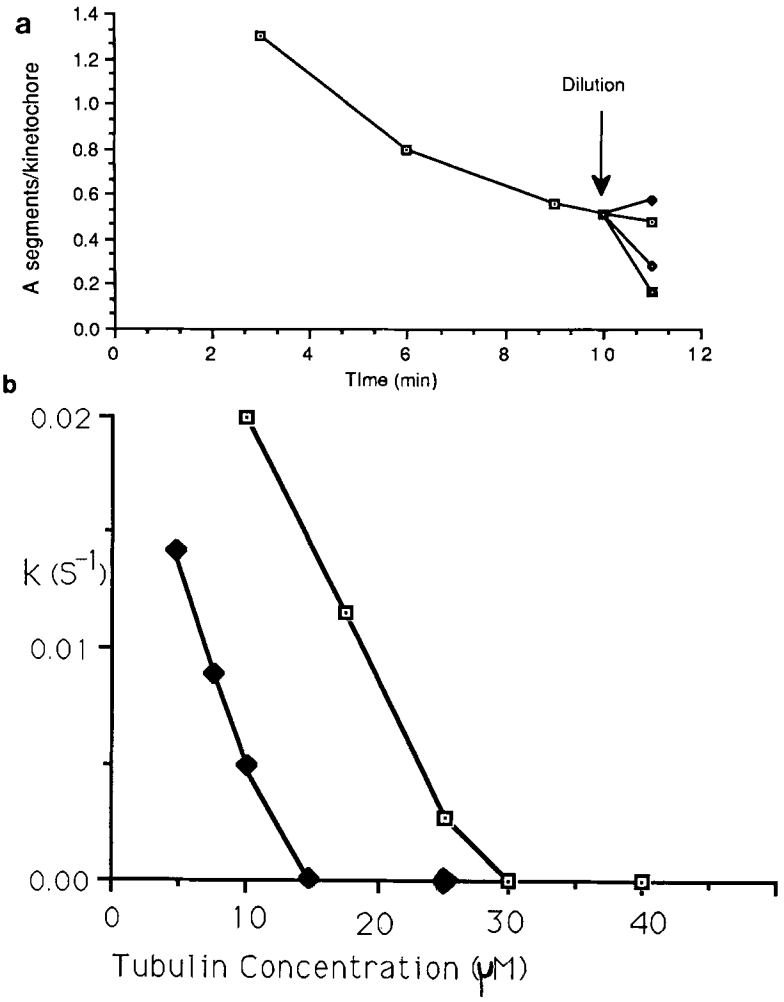

Figure 4. (*a*) Number of A segments per kinetochore after dilution into different tubulin concentrations. Stable labeled seeds were captured by chromosomes and plus end-directed movement was initiated in 25 μM tubulin at time 0 and continued for 10 min as in Fig. 3. NEM-tubulin was added to suppress minus end polymerization (see Materials and Methods). At 10 min the complexes were diluted into various tubulin concentrations, containing one part in four biotin tubulin required to determine transition rates for free microtubules (see Materials and Methods), and fixed after 1 min. Aliquots were processed for immunofluorescence as in Fig. 1. The number of A segments per kinetochore is plotted against time. 30 μM (*closed diamond*); 25 μM (*open square*); 16 μM (*open diamond*); 10 μM tubulin (*closed square*). (*b*) Estimated catastrophe rates for free and kinetochore-attached microtubule plus ends as a function of tubulin concentration. The rates for kinetochore microtubules were estimated from Fig. 4 *a*, assuming that loss of microtubules was a first-order process, and corresponded to catastrophe transitions. Rates for free microtubules were estimated from the fraction which grew continuously during the first minute after dilution to test tubulin concentrations. This fraction was determined by counting microtubules with biotin-labeled tips on their plus ends (see Materials and Methods). Free and kinetochore rates were determined after dilution from the same reaction, ensuring identical conditions. (*open squares*) Kinetochore microtubules; (*filled diamonds*) free plus ends.

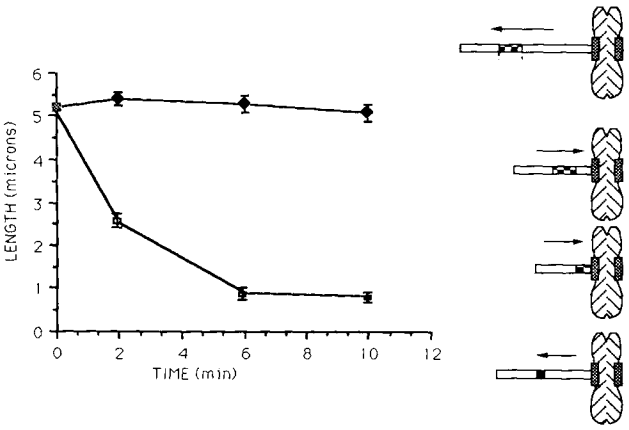

Figure 5. Evidence for rescue transitions in kinetochore microtubules. Unstable labeled microtubule seeds were polymerized using biotin-labeled tubulin and GTP. These seeds have the same stability properties as normal microtubules. The seeds were captured by kinetochores in the presence of sufficient biotin-labeled tubulin to stabilize them. At time zero on the graph, the mixture of complexes and free seeds was diluted into 15 μM unlabeled tubulin, 1 mM GTP, and 1 mM ATP. After fixation at various time points and staining as in Fig. 1 the length of the internal biotin-labeled segment was determined for free microtubules, and the length of Segment B for kinetochore microtubules. Only microtubules displaying a clear A segment were measured. (*a*) (*Open squares*). Average segment B lengths for kinetochore microtubules. (*Filled diamonds*) Average lengths of internal labeled segment for free microtubules from the same reaction. 60–100 microtubules measured per time point. Error bars are standard errors of means. (*b*) Interpretation of this result. The shaded rectangles are kinetochores; the hatched areas internal to the microtubule are the B segments. The arrow shows the direction of movement of the labeled segment with respect to the kinetochore. The microtubule transits from growing to shrinking and the kinetochore initiates minus end–directed movement. Rescue occurs in the middle of the labeled segment, and the kinetochore resumes plus end–directed movement.

of microtubule loss depended strongly on tubulin concentration, increasing at lower concentrations. We interpret this observation as showing that detachment from the kinetochore depends on a catastrophe transition, and thus that the rate of loss of microtubules gives an estimate of the catastrophe rate. We also measured the fraction of free microtubule plus ends that had continued to grow for 1 min after dilution from the same tubes as the kinetochore reactions. This was determined by counting how many microtubules had added a short, biotin-labeled tip onto their plus ends (see Materials and Methods). At 15 μM and above all plus ends grew continuously for 1 min, and thus the catastrophe rate was negligible. Below 15 μM the catastrophe rate increased as tubulin concentration decreased, consistent with previous data (Mitchison and Kirschner, 1985*b*; Walker et al., 1988).

We used the data in Fig. 4 *a* and data for free microtubule plus ends to estimate catastrophe rates at different tubulin concentrations, assuming that microtubule loss was a first-order process. The results are plotted in Fig. 4 *b*. They show that growing plus ends for both kinetochore and free microtubules are stabilized by increasing tubulin concentration as expected, but that kinetochore microtubules undergo catastrophes at much faster rates over the whole concentration range. We should note that our methods for estimating catastrophe rates are certainly less accurate than the direct determinations of Walker et al. (1988), but that they are less laborious, and can probe a greater range of rates.

Rescue of Kinetochore Microtubules

Under conditions of dynamic instability, microtubules that have started shrinking will sometimes rescue and reinitiate growth at a rate that depends on tubulin concentration (Walker et al., 1988). To determine whether kinetochore microtubules underwent rescue events we set up a plus end–directed movement assay in which the labeled B segment was not stabilized by a GTP analogue, and was thus free to depolymerize. A rescue occurring when the microtubule had shrunk back through half of such a labeled segment would result in its shortening, which we could score. We compared the length of B segments for free and kinetochore microtubules during plus end–directed movement at 15 μM tubulin. From the data in Fig. 4, this concentration was expected to result in transitions for kinetochore-attached, but not for free plus ends. The length of the internal labeled segment was constant for free microtubules as expected (Fig. 5 *a*), since they elongate continuously on both ends at 15 μM tubulin. For kinetochore microtubules, however, average B length decreased progressively with time (Fig. 5 *a*), as did the number of B segments per chromosome (not shown). Our interpretation of this result is shown in Fig. 5 *b*. This experiment confirms that kinetochore microtubules transit to shrinking at concentrations where free microtubules do not, and shows that they can also rescue and resume polymerization while remaining attached. We note that in this experiment free and kinetochore microtubules behave in a completely different way, which provides additional confirmation that the dynamics of kinetochore-attached plus ends occur while they remain attached, and not while they are transiently free. These data demonstrate that kinetochore-attached plus ends exhibit the whole range of dynamic instability behavior in vitro. Accurate determination of transition and rescue rates will require real-time analysis.

5′ Adenylylimido Diphosphate (AMPPNP) Induces Rigor at the Kinetochore

We suspected that the kinetochore ATPase responsible for plus end–directed movement might play a role in microtubule destabilization, so we next investigated the effect of a nonhydrolyzable ATP analogue on kinetochore microtubule dynamics. When AMPPNP was added during polymerization at the kinetochore, plus end movement stopped immediately (Fig. 6). This suggested that AMPPNP induces a rigor state in the kinetochore ATPase thought to be responsible for plus end–directed movement. AMPPNP-induced rigor has been observed previously with kinesin, another microtubule motor (Vale, 1987). Furthermore, the number of A segments per kinetochore, which decreases during plus end–directed movement at 25 μM tubulin (Fig. 4 *a*), did not decrease any further after addition of AMPPNP (data not shown). When such rigor complexes were diluted (again glycerol was added to slow down depolymerization), we found that the length of A segments per kinetochore remained almost constant for 10 min (Fig. 6), as did their number (data not shown) after the subsequent dilution. This stabilization of A segments could be explained in two ways: either the catastrophe is markedly inhibited by the rigor state, or else minus end–directed movement itself is inhibited by AMPPNP.

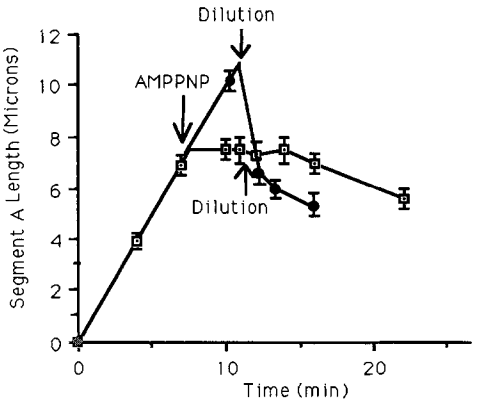

Figure 6. AMPPNP inhibits plus end-directed movement and stabilizes kinetochore microtubules. Plus end-directed movement was initiated as in Fig. 2. The reaction mixture was split in two, and 5 mM AMPPNP and 5 mM $MgCl_2$ were added to one aliquot at 7.5 min. At 10 min the control aliquots were diluted 1:20 into buffer containing 12% glycerol and no nucleotides (as per Fig. 2). At 10.5 min the AMPPNP aliquot was diluted in the same way. 80–130 A segments were measured per time point. Error bars are standard error of the mean. (*Open squares*) Average segment A length for AMPPNP aliquot. (*Filled circles*) Average segment A length for control aliquot.

To distinguish direct effects of AMPPNP on minus end-directed movement from effects on catastrophe rate, we added AMPPNP either shortly before or after dilution. In the latter case the catastrophe should already have occurred before addition of the analogue. When AMPPNP was added 30 s after dilution we observed no stabilization of A segments, and minus end-directed movement continued (Fig. 7). Separate experiments showed no perceptible effect of AMPPNP on the decrease of average A length with time when added after dilution, compared to control dilutions in which no AMPPNP was added (not shown). When AMPPNP was added 30 s before dilution in the same experiment, it stabilized the A segments (Fig. 7). We conclude that the AMPPNP-induced rigor state prevents induction of minus end-directed movement, but does not inhibit it if it had already started. Thus, growing and shrinking ends must interact differently with the kinetochore.

Discussion

Microtubules exist as a dynamic population in which they both grow and shrink, and transit between these two states, a behavior termed dynamic instability. Since microtubules retain dynamic properties when attached to kinetochores, and the movement of chromosomes at mitosis is closely linked to the changing length of kinetochore microtubules, control of microtubule dynamics at the kinetochore could play a crucial role in chromosome movement. In this paper we have addressed this problem by determining the influence of kinetochores on the dynamic behavior of their attached microtubules in vitro. We have found that microtubule plus ends exhibit the full range of dynamic instability behavior while remaining attached to kinetochores, but at altered transition rates compared to free ends. Furthermore, our data suggest that a kinetochore ATPase may be able to modulate transition rates, though whether it does so under physiological conditions is not clear.

Implications of Microtubule Destabilization by Kinetochores

The specific regulation that we have discovered in vitro is that kinetochores markedly increase the catastrophe rate of attached microtubules. The extent of this destabilization is such that kinetochore microtubules undergo frequent catastrophes at tubulin concentrations which completely stabilize free plus ends. This observation directly contradicts the previous observation of stabilization of microtubules nucleated by centrosomes described previously (Mitchison and Kirschner, 1985*b*). In that study the number concentration of centrosome-kinetochore complexes was small, and the length of the stable microtubules was not quantitated. With the benefit of hindsight it seems most likely that those stable complexes corresponded to a small minority of captured plus ends that do not initiate depolymerization. Such apparently stable complexes were observed in the experiments reported here, and in Koshland et al. (1988), but they represent only a small fraction of the population of kinetochore-attached plus ends. Their existence tends to skew length distributions to longer lengths at long times after dilution, see for example Fig. 3, and the long remaining microtubules in Fig. 2 *d*. Whether this stable subset is simply artifact or has some physiological significance is not known. AMPPNP-induced rigor causes stabilization of all kinetochore-attached plus ends (Fig. 6), and it is possible that the absence of ATP induced some degree of rigor in the centrosome-kinetochore complexes reported in 1985.

If kinetochores promote catastrophes in attached microtubule plus ends in living cells, it would tend to make kinetochore microtubules initiate depolymerization more often than astral microtubules. This seems paradoxical given the weight of evidence showing that kinetochore microtubules

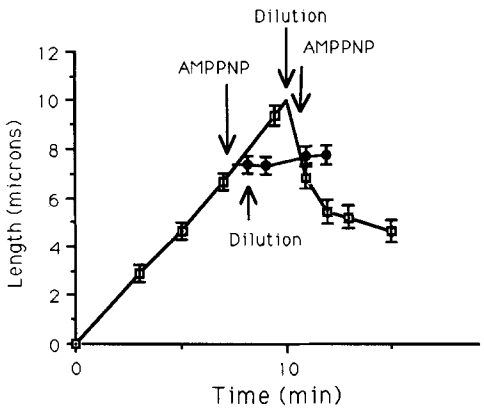

Figure 7. AMPPNP inhibits the catastrophe transition for kinetochore microtubules, but does not inhibit minus end-directed movement of kinetochores. Stable labeled seeds were captured and plus end-directed movement was initiated as in Fig. 2. The reaction mixture was split in two. To one aliquot, 5 mM AMPPNP and 5 mM $MgCl_2$ was added at 7.5 min, followed by 1:20 dilution into buffer containing 12% glycerol and no nucleotides 45 s later. The other aliquot was diluted 1:20 into buffer containing 12% glycerol and no nucleotide at 10 min, and 15 s later the same amount of AMPPNP was added. Aliquots from both reactions were fixed at the indicated times and processed as in Fig. 1. 60–130 A segments were measured per time point. Error bars are SEM. (*Open squares*) AMPPNP added before dilution. (*Closed circles*) AMPPNP added after dilution.

turn over more slowly and are more resistant to depolymerization (see introduction). However, the paradox is resolved if we distinguish between catastrophe transitions of ends while remaining attached to the kinetochore from catastrophe transitions resulting in detachment. Kinetochore microtubules are now known to turn over in vivo (Wadsworth et al., 1989), so catastrophes with detachment can occur, but in general kinetochore microtubules do not depolymerize unless the kinetochore is free to follow their shrinking ends as in anaphase. Thus, kinetochores may promote catastrophe transitions yet still stabilize attached microtubules against turnover, because the chromosomes are not always free to move polewards. On the basis of the data in this paper, we suggest that kinetochores continuously induce catastrophe transitions of their attached microtubules, but that this generally does not result in depolymerization unless the kinetochore can move polewards, following the shrinking end.

During anaphase, promotion of catastrophe transitions by the kinetochore alone could account for the observation that kinetochore microtubules shorten while astral microtubules grow (Bajer and Mole-Bajer, 1972). During metaphase the paired kinetochores are oriented to opposite poles and thus cannot move polewards. This prevents any catastrophes from translating into net depolymerization, except for the transient depolymerization that occurs during oscillations. If this constraint is removed by ablation of one kinetochore of a pair during metaphase, the remaining kinetochore immediately moves polewards (McNeill and Berns, 1984), presumably with depolymerization at the kinetochore. During prometaphase, capture of a plus end by the kinetochore may not in fact stabilize the end against catastrophe as previously proposed (Mitchison and Kirschner, 1985*a*), but rather catastrophes must now be coupled to chromosome movement. Thus, the captured microtubule is stabilized as a kinetochore microtubule not because it cannot undergo a catastrophe, but rather because it cannot detach. This is a much more realistic picture of kinetochore fiber formation than an earlier static model (Kirschner and Mitchison, 1986). The reader is referred to Rieder et al. (1989) for the most recent information on prometaphase capture. Thus, it is plausible that kinetochores may destabilize attached microtubules throughout mitosis.

This extrapolation to living cells is highly speculative since the in vitro assay is far from mimicking all aspects of kinetochore microtubule behavior in the spindle. In particular, the rate of depolymerization in vitro is much faster than physiological, and detachment of microtubules from the kinetochore is much faster in vitro. This may mean that we have failed to reconstitute the true microtubule-kinetochore interaction, or rather that kinetochore microtubules are stabilized by other factors, such as associated proteins, in the spindle. Our hypothesis is important, however, in that it can explain aspects of the unique dynamic behavior of kinetochore microtubules without postulating any unique associated proteins or other modifications. A second problem with the in vitro assay is that we do not yet know whether useful force is generated during plus or minus end-directed movement. Further experiments involving real time observation of movement and measurement of forces are in progress to address such questions.

Currently there is much debate about the mechanism of force generation for polewards chromosome movement (Koshland et al., 1988; Rieder, 1989). However, there is general agreement that microtubule depolymerization governs the rate of polewards movement once the kinetochore fiber is established (Begg and Ellis, 1979; Nicklas, 1983). It is thus quite possible that dynamic instability at the kinetochore could provide the information telling the kinetochore which direction to move, even if it turns out that ATPases provide the motive force.

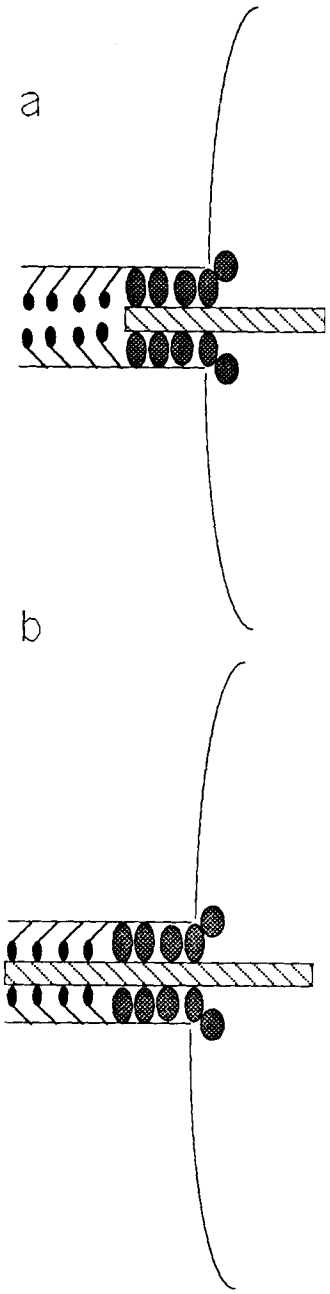

Figure 8. Model for a spatially segregated kinetochore. The kinetochore is drawn as a collar around the microtubule end. The tadpoles represent ATPase molecules responsible for plus end–directed movement. The molecules that hold onto the shrinking end of microtubule are drawn as ovals, and are in a more external position in the kinetochore. The kinetochore in *b* is undergoing plus end–directed movement on a growing microtubule. The ATPase molecules engage the lattice, and AMPPNP addition would induce rigor. The holding-on molecules slide over the lattice. The kinetochore in *a* is undergoing minus end–directed movement on a shrinking microtubule. The ATPase molecules do not engage the lattice, and AMPPNP addition would have no effect. The holding-on molecules cause the kinetochore to follow the shrinking microtubule end.

Mechanism of Destabilization by Kinetochores

Once the microtubule lattice has hydrolyzed its GTP (Weisenberg and Deery, 1976), it is thermodynamically poised to depolymerize. Thus, the kinetochore need only catalyze the transition process. Currently we do not know what makes a free microtubule end initiate depolymerization, let alone

one attached to a kinetochore. The AMPPNP results suggest that a kinetochore ATPase, presumably the same one responsible for plus end–directed movement, could play some role in catalyzing the transition. Alternatively, kinetochores could destabilize simply by their inhibition of the polymerization rate. The complications of frequent transitions make it difficult for us to accurately measure polymerization rate at the kinetochores in the assays described here. However, it seems to be slower than that for free plus ends (Fig. 3). According to the GTP cap hypothesis, slower growth leads to a smaller cap, and a greater probability of loosing the cap (Kirschner and Mitchison, 1986). Since polymerization of kinetochore microtubules is relatively slow in metaphase cells (Mitchison, 1989; Mitchison et al., 1986), the same mechanism could apply in vivo.

Implications for Kinetochore Structure

One of the interesting features exhibited by kinetochores during microtubule-based movement in vitro, is that the two different directions of movement, minus and plus end–directed, have very different characteristics. Plus end–directed is an ATP-dependent (Mitchison and Kirschner, 1985*a*) movement along the microtubule lattice, whereas minus end–directed is an exogenous nucleotide-independent movement that follows the shrinking end of a microtubule (Koshland et al., 1988; Mitchison and Kirschner, 1985*a*). In this paper we have shown that the kinetochore can switch between the two directions of movement on the same microtubule. How is the kinetochore organized so that these two activities do not oppose each other? An important clue comes from the effect of adding AMPPNP. If added during plus end–directed movement, it induces rigor and stabilizes microtubule ends against depolymerization, while addition during minus end–directed movement has no effect. We suggest that the two different motors in the kinetochore are spatially segregated, with the ATPase molecules occupying a more internal position, as cartooned in Fig. 8. This was previously proposed theoretically (Huitorel and Kirschner, 1988; Mitchison, 1988). This model can explain many aspects of kinetochore behavior in in vitro assays, and it also has the interesting feature that external force on a microtubule could control how it interacts with the kinetochore, by influencing the depth of penetration.

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