The Polarity and Stability of Microtubule Capture by the Kinetochore

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Abstract. We have studied the capture of microtubules by isolated metaphase chromosomes, using microtubules stabilized with taxol and marked with biotin tubulin to distinguish their plus and minus ends. The capture reaction is reversible at both the plus and minus ends. The on rate of capture is the same for both polarities but the dissociation rate from the kinetochore is seven times slower with microtubules captured at their plus ends than those captured at their minus ends. At steady state this disparity in off rates leads to the gradual replacement of microtubules captured at their minus ends with those captured at their plus ends. These results suggest that the kinetochore makes a lateral attachment near the end of the microtubule in the initial capture reaction and shows a structural specificity that may be important in proper bipolar attachment of the chromosome to the spindle.

MITOTIC and meiotic chromosomes are attached to the spindle by a specialized structure known as the kinetochore (Rieder, 1982; Ris and Witt, 1981). Little is known about the kinetochore: its composition, its mode of attachment to microtubule, or its role in chromosome movement during prophase and anaphase. It is known that the kinetochore is associated with a unique region of DNA to which is presumably attached specific proteins that allow that region and only that region to interact with the microtubules of the spindle.

Genetic experiments in yeast have shown that this specialized region of DNA can be very small, but these experiments have not yet answered how many other, presumably proteinous (Cox et al., 1983; Earnshaw and Rothfield, 1985; Valdivia and Brinkley, 1985), components must be present to mediate the interaction with microtubules. Therefore, most of what has been learned about kinetochores has been through the extensive study of the behavior of chromosomes during mitosis or meiosis (Alberts et al., 1983; Inoue, 1981; McIntosh, 1984; Murray and Szostak, 1985; Pickett-Heaps, 1986; Pickett-Heaps et al., 1982). These studies are generally descriptive, though some have used pharmacological agents, micromanipulation, or permeabilized cell systems (Bajer et al., 1982; Brinkley et al., 1967; Nicklas, 1983; Cande et al., 1981; DeBrabander et al., 1981; Pepper and Brinkley, 1979).

Two questions have dominated recent studies of mitosis. First, the origin of microtubules in the spindle, in particular those attached to the kinetochore (Bergen et al., 1980; De-Brabander, 1982; Euteneuer and McIntosh, 1981; McDonald et al., 1979; Nicklas and Gordon, 1985; Telzer and Haimo,

1981). Second, how do the microtubules lengthen in prophase when chromosomes may move away from the poles and shorten in anaphase when the chromosomes move toward the poles. Recently some of these questions have been investigated by microinjection of labeled subunits into the spindle and subsequent visualization of the sites of new subunit addition during metaphase and into anaphase (Mitchison et al., 1986). Microtubules seem to be continuously nucleated at the centrosome, from which most of the microtubules originate. Tubulin subunit insertion into microtubules comprising the kinetochore fibers takes place during metaphase at the kinetochore, while most subunit loss occurs at the kinetochore during anaphase. Recently the anaphase loss at the kinetochore also has been supported by photobleaching experiments (Gorbsky et al., 1987). Although the role of net positive flux of subunits at metaphase is still unclear, these experiments demonstrate that both positive and negative fluxes can occur at the kinetochore while the kinetochore still retains its attachment to microtubules. These in vivo experiments suggest that kinetochore microtubules arise by capture and that once captured, growth or shrinking can occur at the kinetochore. What is unclear about the initial reaction is how the kinetochore captures microtubules. Does it, for example, make a lateral attachment or an end-wise attachment? It is also unclear what generates the fluxes and the accompanying forces. We will deal with the question of the mode of the initial attachment of microtubule to kinetochore in this report.

It has recently been possible to study the interaction between microtubules and the kinetochore in vitro (Mitchison, 1985a). The kinetochore region of mitotic chromosomes efficiently captures microtubules. It will also nucleate microtubules, but the nucleation reaction is inefficient in that it requires a concentration of free tubulin close to the level needed for spontaneous assembly in vitro. Nucleation by the

centrosome by contrast is very efficient and will support some measurable assembly at concentrations of tubulin four-fold lower than the bulk critical concentration. The microtubules nucleated by the centrosome are individually unstable but if chromosomes are present the distal end may be captured by the kinetochores and stabilized. Thus, the in vitro experiments strongly support a model for the generation of kinetochore microtubules in which centrosomes nucleate microtubules at random and chromosomes capture and stabilize them (Hill, 1985; Kirschner and Mitchison, 1986).

How is the capture and stabilization achieved? Capture seems to occur with either polarity suggesting that either end-wise interactions can occur at either end or that the interaction is basically lateral and independent of the ends. Further evidence for lateral interaction has come from studying translocation reactions of microtubules attached to chromosomes. In the presence of ATP and tubulin, subunits can be inserted proximal to the kinetochore and the microtubule segments with the proper polarity translocated away from the kinetochore, a reaction probably related to prophase movements where chromosomes move away from the poles (Mitchison, 1985). However, under some conditions polymerization and translocation can be unlinked and microtubules captured by the kinetochore can be elongated and subsequently translocated by addition of ATP. Under these circumstances the kinetochore microtubules must make a lateral interaction with the kinetochore; however, it is not clear whether the initial interaction with the kinetochore microtubules is lateral or end-wise.

In this report, we study in detail the polarity and stability of microtubules captured by the kinetochores. By using newly developed microtubule substrates for distinguishing the polarity of capture, we show that the initial capture reaction still allows for attachment and detachment of microtubule ends. The detachment reaction is slower for plus ends, suggesting that the kinetochore can distinguish microtubule polarity. The initial capture reaction shows no ATP dependency, whereas subsequent translocation does. We propose that microtubules are initially captured at or near their ends by a process not requiring energy.

Materials and Methods

Chromosome Isolation

Mitotic chromosomes were isolated from Chinese hamster ovary (CHO) cells arrested with a 10 µg/ml vinblastine sulfate overnight, by a procedure (Mitchison and Kirschner, 1985a) adapted from that of Lewis and Laemmli (1982) with a few modifications as follows. Mitotic cells were collected from 10 dishes and pelleted at 500 g for 5 min. The pellets were resuspended at 4°C (temperature at which all subsequent steps were done) in 50 ml of swelling buffer composed of 5 mM Pipes, 10 mM NaCl, 5 mM MgCl₂, 0.5 mM EDTA, pH 7.2 with KOH. After a 10-min incubation, the cells were pelleted again for 3 min and resuspended very gently in 3 ml of lysis buffer saturated with digitonin and containing 20 mM Pipes, 2 mM EDTA, 1 mM spermidine HCl, 0.5 mM spermine HCl, 0.1% β-mercaptoethanol, 2 µg/ml α₂ macroglobulin (added just before use), pH 7.2 with KOH. After 2 min, the permeabilized cells were sedimented at 500 g for 3 min, resuspended again in 3 ml of lysis buffer and transferred to a glass Dounce homogenizer. The cells were homogenized by 10 strokes with the loose pestle and the resulting mitotic figures were further disrupted by ∼40 strokes with the tight pestle. The chromosome morphology was followed under a fluorescence microscope during the homogenization by removing aliquots diluted with an equal volume of lysis buffer containing 5 µg/ml Hoechst 33 258 dye.

The total lysis and homogenization step took ∼10 min. The unbroken mitotic figures were removed by sedimentation at 250 g for 1 min and the su-

pernatant was then layered on a sucrose step gradient: a 2 ml 60% sucrose (wt/vol), 2 ml 50% sucrose, and 5 ml 30% sucrose, all containing 10 mM Pipes, 1 mM EDTA, 0.5 mM spermidine HCl, 0.25 mM spermine HCl, 0.1% β-mercaptoethanol, 1 µg/ml α₂ macroglobulin, pH 7.2 with KOH. The step gradient was centrifuged at 2,500 g for 15 min in a JS 13 (Beckman Scientific Instruments, Palo Alto, CA) swinging bucket rotor. The chromosomes formed a white mass at the 50–60% sucrose interface, and were collected with a Pasteur pipette after careful aspiration of the top of the gradient. 50-µl aliquots were frozen in liquid nitrogen and stored at −75°C until use.

Tubulin and Biotinylated Tubulin Preparations

Phosphocellulose-purified tubulin (PC-tubulin)[1] was prepared from bovine brains by a modification of the procedure of Weingarten et al. (1975) as described by Mitchison and Kirschner (1984). Protein concentrations were determined by the method of Bradford (1976) using BSA as a standard. Biotinylated tubulin (Mitchison and Kirschner, 1985a) was prepared as described by Kristofferson et al. (1986).

Preparation of Microtubules used in the Kinetochore Capture Assays

According to the type of experiment to be performed, microtubules were polymerized in different ways but always from PC-tubulin (without microtubule associated proteins).

Microtubules were assembled at 37°C from PC-tubulin in Pipes buffer (P buffer; 80 mM Pipes, 1 mM EGTA, 1 mM MgCl₂, pH 6.8, with KOH) and 1 mM GTP, then added to chromosomes, the final soluble tubulin concentration being between 10 and 15 µM, close to the critical concentration under these conditions.

Alternatively, microtubule seeds were made from PC-tubulin in P glycerol Mg buffer (30% vol/vol glycerol, 80 mM Pipes, 1 mM EGTA, pH 6.8, and either 1 mM GTP and 5 mM MgCl₂, or 4 mM GTP₇S and 8 mM MgCl₂) at 37°C for 20 min if GTP was present or for 90 min if GTP₇S was present.

Shorter seeds at a higher number concentration were produced by multiple pipetting during the assembly reaction. This procedure was used either to make seeds from unmodified PC-tubulin or to make biotinylated seeds from biotinylated PC-tubulin. These seeds were used directly or further processed as follows before addition to chromosomes.

For experiments where it was important to know the polarity of individual microtubules, segmented microtubules with unlabeled tubulin at both ends were made as follows. Biotinylated seeds, made in glycerol buffer as described above, were elongated for 10–15 min at 37°C by an 8–10-fold dilution into unmodified PC-tubulin at 2 mg/ml in Pipes buffer, conditions favorable to the elongation but not to the nucleation of microtubules. Segmented microtubules were also made with unmodified tubulin cores by elongating seeds made of unmodified PC-tubulin in biotinylated tubulin.

Another substrate that was very useful for polarity experiments was a preparation of microtubule seeds with unlabeled growth on only the plus end. These seeds were prepared by blocking minus end assembly with NEM tubulin (Mitchison and Kirschner, unpublished observations). PC-tubulin was modified by reaction with 1 mM N-ethylmaleimide at 4°C in P buffer for 5 min and the reaction was quenched by β-mercaptoethanol. The N-ethylmaleimide-modified tubulin (NEM) did not polymerize by itself in P buffer and prevented elongation at the minus end of microtubules without affecting the elongation rate at the plus end when used at the appropriate stoichiometry of NEM tubulin vs. unmodified tubulin (Mitchison and Kirschner, unpublished observations).

To prepare seeds with unlabeled growth at one end, biotinylated seeds were elongated as described above by dilution in Pipes buffer at a final concentration of 15 µM PC-tubulin and 5 µM NEM tubulin for 15 min at 37°C. These could then be used directly for capture experiments. The various kinds of microtubule products described were either used immediately or were stabilized by a gradual addition of taxol up to a final concentration of 10 µM. These could then be distributed by careful pipetting, using cut off pipette tips into 5- or 10-µl aliquots, frozen in liquid nitrogen and kept at −75°C. Aliquots would then be thawed just before use and incubated with chromosomes in Pipes buffer adjusted to a final concentration of 4 µM taxol

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1. *Abbreviations used in this paper*: NEM tubulin, N-ethylmaleimide-modified tubulin; PC-tubulin, phosphocellulose-purified tubulin; P-buffer, Pipes buffer.

to prevent microtubule disassembly. These extract conditions avoided problems with microtubule bundling during fixation by glutaraldehyde.
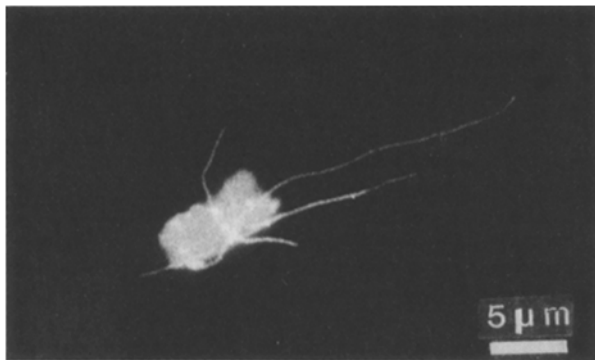
Capture

The capture of microtubules was carried out essentially as previously described (Mitchison and Kirschner, 1985b). Microtubules stabilized by 10 μM taxol were thawed and diluted ~10-fold into P buffer at 37°C in the presence of 4 μM taxol, 1 mM GTP, and 1 mM ATP-Mg or 1 mM UTP-Mg whenever necessary. After 1 min at 37°C, chromosomes were added at the desired concentration (between 10 and 50% of the final incubation mixture). At all stages of the capture experiments, 4 μM taxol was present to stabilize microtubule without inducing bundling in these conditions. The final volume of the reaction mixture was between 50 and 100 μl, and 10-μl aliquots were fixed at different time intervals and processed as described below.

Fixation and Visualization of Microtubules and Microtubule-Chromosome Complexes
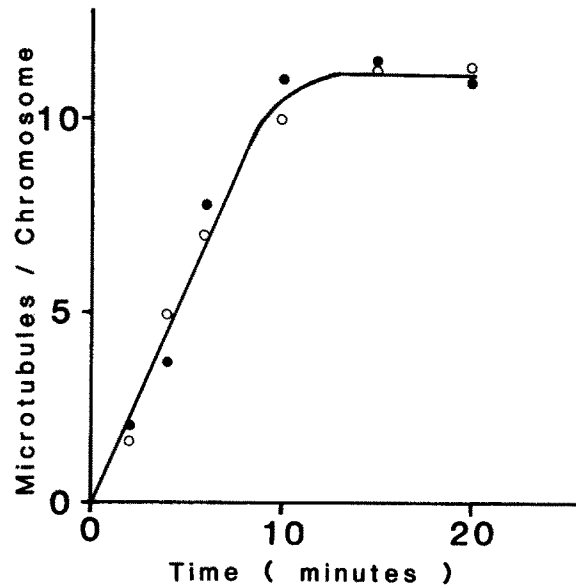
We found that the nonspecific cross-linking of free microtubules in solution to the arms of chromosomes could be kept to a minimum if the final glutaraldehyde concentration was 0.2% and the fixation time was 1 min. This did not affect the efficiency of fixation of microtubule and microtubule-chromosome complexes (control experiments using 1% glutaraldehyde for 3 min showed the same length distribution in free microtubule populations). Samples were routinely fixed for 1 min at 30°C by a 20-fold dilution in prewarmed P buffer containing 0.20% glutaraldehyde, then further diluted (according to the final desired density) by buffer at 4°C and kept on ice. When only microtubule-chromosome complexes were to be observed the samples were sedimented onto poly-L lysine–coated coverslips through a 5-ml cushion made of 40% glycerol in P buffer in modified Corex tubes spun at 16,000 g for 10 min as previously described (Evans et al., 1985). Alternatively if we wished to observe all microtubules in solution, the samples were extensively diluted and sedimented directly (without cushion) on to coverslips at 27,000 g for 30 min.

The sedimented material was then postfixed in methanol at −20°C for 5 min, washed in PBS and processed for immunofluorescence as described (Mitchison and Kirschner, 1985a) using monoclonal antibodies against α- or β-tubulin (a generous gift from Dr. S. Blose), rabbit anti–biotin antibodies from Enzo Biochemical, Inc., New York, NY). FITC goat anti-mouse IgG and RITC goat anti-rabbit IgG antibodies (from Cappel Laboratories, Malvern, PA), and the Hoechst 33258 dye.

The quantitation of the experiments was done as follows, using no less than 100 chromosomes with their connected microtubules per time point. Captured microtubules were counted directly in the fluorescence microscope. Whenever length measurements were necessary, we took pictures of the microtubules or the complexes using the appropriate fluorescence filters (fluorescein, rhodamine, Hoechst) on Kodak Tri-X Pan film processed in Diafine. Negatives were directly projected through an enlarger on a digitizer

Figure 2. Kinetics of capture. Chromosomes were allowed to interact with stable microtubules in the presence of 4 μM taxol with (●) or without (○) 1 mM ATP-Mg. After different incubation times at 37°C, aliquots were fixed, processed for immunofluorescence, and the number of microtubules interacting with kinetochores was determined.
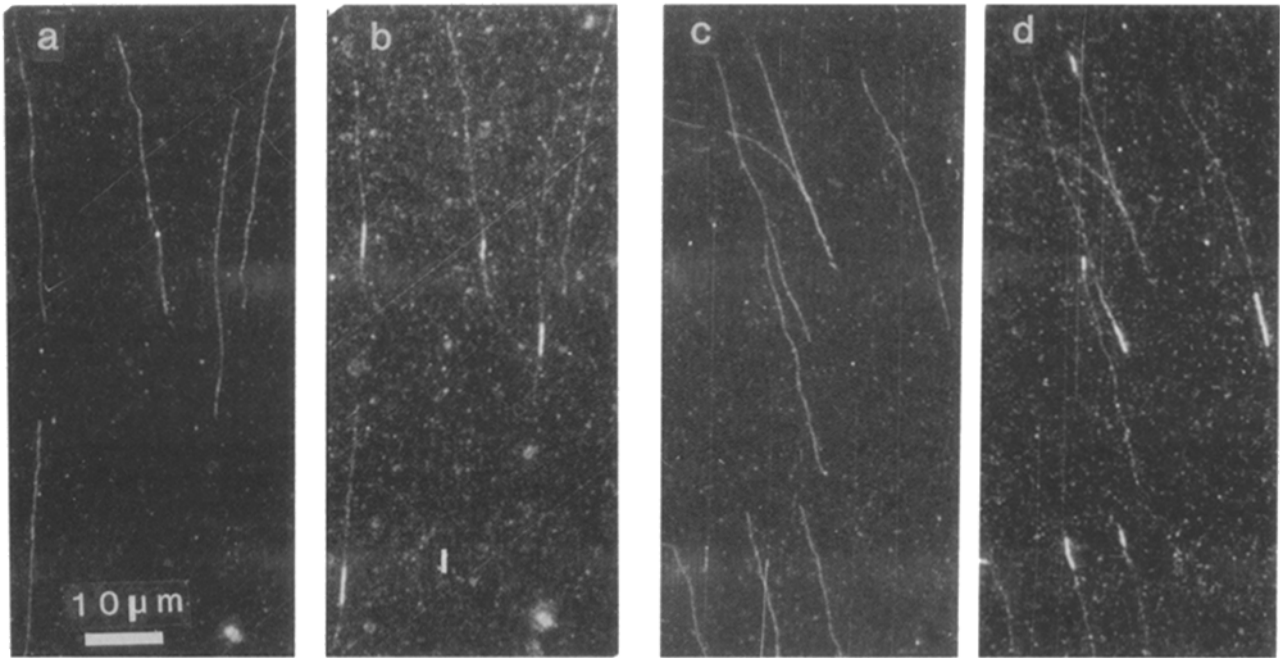
connected to a computer. Microtubule segments (labeled, unlabeled, total length) were traced and digitized with the pen for each free or captured microtubule. The data were stored and processed as described (Kristofferson et al., 1986).

Results

Preparation of Stable Microtubules For Use in the Capture Assay and the Kinetics of Capture

Capture is defined as the attachment of a microtubule to the constricted portion of the chromosome, as assayed by fluorescence microscopy. We found previously that isolated chromosomes will capture microtubules in vitro (Mitchison and Kirschner, 1985b); however, in these studies the polymer number and average length changed rapidly during the course of the experiment due to dynamic instability of the microtubules (Mitchison and Kirschner, 1984b). Therefore, to develop a more controlled capture assay we have suppressed the dynamics by stabilizing microtubules with taxol under conditions that neither induced significant amounts of abnormal polymeric forms of tubulin nor interfered with binding to the kinetochore. The taxol-stabilized microtubules could be aliquoted and stored frozen. The length distribution of microtubules after freezing and diluting (11.9 ± 0.5 μm) was identical to that observed before freezing (12.1 ± 0.5 μm).

To test whether taxol-stabilized microtubules were good substrates for the capture assay, we mixed chromosomes with stable microtubules in our standard capture conditions for various periods of time at 37°C, fixed aliquots, and analyzed them by immunofluorescence. As previously described for dynamic microtubules (Mitchison and Kirschner, 1985b), stable microtubules appeared to bind to the kinetochore region of the chromosomes (Fig. 1).

Figure 1. Immunofluorescence picture of stable microtubules captured by kinetochores. Microtubules stabilized by 10 μM taxol were incubated with chromosomes in our standard assay for capture (see Materials and Methods) in P buffer containing 4 μM taxol for 10 min at 37°C. An aliquot was fixed and processed for immunofluorescence to visualize microtubules using monoclonal anti-β tubulin antibodies and FITC goat anti-mouse IgG antibodies.

Figure 3. Immunofluorescence of free stable microtubules containing an internal polarity marker. Biotinylated tubulin was polymerized in the presence of GTPγS in glycerol-Mg buffer to produce stable microtubule seeds. Those seeds were then diluted 100-fold either into 14 μM regular tubulin (*a* and *b*), or into a mixture of 5 μM NEM tubulin and 14 μM regular tubulin (*c* and *d*) in P buffer containing 1 mM GTP and no glycerol. The samples were then fixed and processed for immunofluorescence. (*a* and *c*) Fluorescein channel showing the antitubulin staining; (*b* and *d*) rhodamine channel showing the antibiotin staining.

As shown in Fig. 2 the capture of taxol-stabilized microtubules reached saturation by 10 min after initiation of the reaction at a microtubule concentration of 1.2×10^{-10} M (7.2×10^{10} microtubules/ml). The capture kinetics were the same in the presence or absence of ATP. Similar kinetics and saturation had been previously observed with dynamic microtubules (Mitchison and Kirschner, 1985*b*). Previously the slow down and plateauing of binding was attributed to dynamic instability of the microtubule population which would cause a decrease in rate of binding due to a decrease in number and increase in length of the microtubules. The observation that similar kinetics are observed with stable seeds implies that the apparent saturation is a property of the kinetochore.
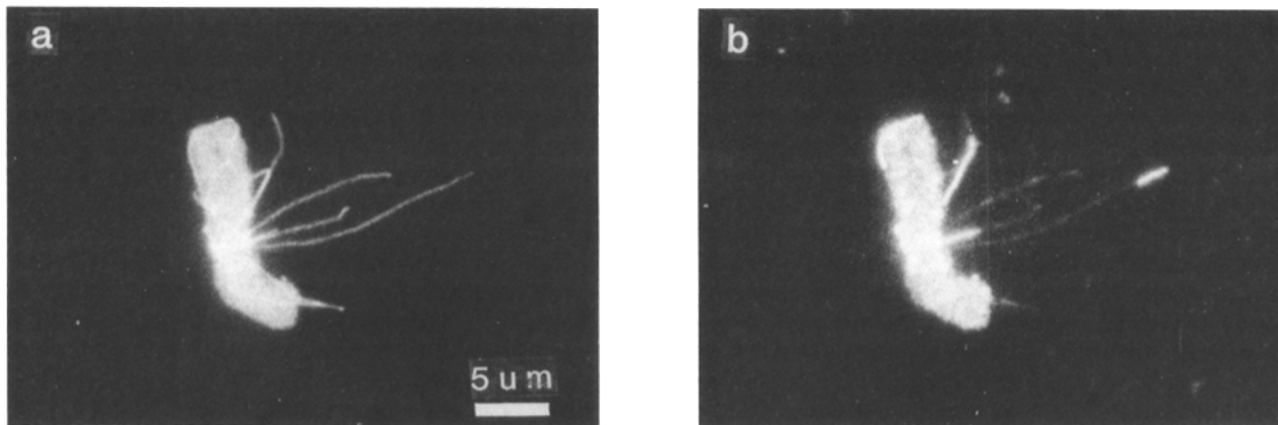
Internal Markers for Microtubule Polarity

Stable markers directly on the microtubule were developed for the study of the polarity of capture and release of microtubules from the kinetochore. Two approaches were used to obtain such polarity markers. In the first, polarity could be assessed by the different lengths of unlabeled tubulin grown off biotin tubulin seeds. The greater growth rate from the plus end as compared to the minus end (1.36 μm/min for the plus vs. 0.33 μm/min for the minus at a concentration of tubulin of 14 μM) led to the formation of nonbiotin segments four times larger on the plus end than on the minus end (Fig. 3, *a* and *b*). These chimeric microtubules could be stabilized with taxol and frozen. A disadvantage of this marker was that shear or stochastic variation could affect the lengths of unlabeled segments and lead to a misidentification of the polarity of the microtubule.

A more satisfactory polarity marker uses chimeric micro-

tubules with label at one end. They were produced by using NEM tubulin to selectively block growth from the minus end (Mitchison and Kirschner, unpublished observations). At 5 μM NEM tubulin and 14 μM unmodified tubulin growth at the minus end is inhibited more than 90% (0.026 μm/min as compared with 0.33 μm/min) but there is no inhibition of growth from the plus end. Under these conditions nonbiotin segments are 50 times longer on the plus end, which makes them essentially undetectable on the minus end (Fig. 3, *c* and *d*).

Since exposure to NEM tubulin could irreversibly alter some property of microtubules at their minus end, and therefore introduce a potential bias in our capture experiments, we examined the rate of elongation after exposure to NEM tubulin. Biotinylated seeds were diluted into unmodified tubulin, stabilized with taxol (Fig. 3, *a* and *b*), and then diluted 20-fold into 14 μM unmodified tubulin. In a parallel experiment, an aliquot of biotinylated seeds was first incubated in a mixture of NEM tubulin and unmodified tubulin and then diluted into unmodified tubulin and incubated further. The growth of the unmodified segments was then measured with the faster growth assigned to the plus end. Both seeds, whether exposed to NEM tubulin or not, showed the same elongation rate at the + end (1.34 ± 0.05 μm/min^{-1} for each). Both showed essentially the same elongation rate at the minus ends 0.34 ± 0.02 μm/min^{-1} (elongated in the absence of NEM tubulin) vs. 0.33 ± 0.02 μm/min^{-1} (elongated in the presence of NEM tubulin). Thus, no effect of NEM tubulin persisted in terms of microtubule growth and therefore once NEM tubulin is removed the microtubule ends have the same kinetic characteristics as untreated ones.

Figure 4. Immunofluorescence of captured stable microtubules containing an internal polarity marker. Stable biotinylated seeds were elongated in the presence of NEM tubulin and regular tubulin as shown in Fig. 3 (*c* and *d*). They were incubated in the presence of chromosomes for 10 min at 37°C, then fixed and processed for immunofluorescence. (*a*) Anti-tubulin staining. (*b*) Anti-biotin staining.

## The Polarity and Reversibility of Microtubule Capture

To assess the polarity of capture, segmented microtubules generated either by elongation of biotinylated seeds in the presence of a mixture of NEM tubulin and unmodified tubulin or by elongation in the absence of NEM tubulin were incubated with chromosomes at 37°C for various periods of time, fixed, and processed for immunofluorescence. An example of the results with segmented microtubules made in the presence of NEM tubulin is shown on Fig. 4.

To obtain more precise estimates of the number of captured microtubules we decreased the concentration of microtubule segments so that on an average a chromosome captures only ∼2.5 microtubules per chromosome, as compared with ∼12 in Fig. 2. As shown in Fig. 5 *a*, a plateau in total number of microtubules captured is again reached in 10 min. In about half of these microtubules it is difficult to assess polarity due to bending of the microtubules or the obscuring effect of the chromosomes themselves. Among the remaining microtubules the number that were captured at their plus ends equaled the number captured at the minus end for the first 10 min (cf. Fig. 5 *b* to 5 *c*, or shown as the ratio of plus to minus in Fig. 5 *d*); After 10 min the ratio of plus to minus ends captured increased (Fig. 5 *d*). The change in the ratio was due to the continued binding of plus end microtubules (Fig. 5 *b*) while there was net loss of minus end microtubules (Fig. 5 *c*). Similar results were obtained with microtubule seeds elongated in the absence of NEM tubulin (not shown) where polarity was estimated from the lengths of the segments. The agreement of both types of analysis suggest that there is no bias in determining plus or minus ends.

These data suggested that the capture reaction must be reversible to allow the total polymer number to remain fixed and yet allow the composition to change. To demonstrate the reversibility of capture, the chromosome microtubule complexes were diluted soon after reaching the plateau, always in the presence of 4 µM taxol. To make analysis more precise again the number of microtubules bound per chromosome was kept low by using a low microtubule concentration in the initial capture reaction. As shown in Fig. 6 after dilution, the number of microtubules bound per chromosome decreases
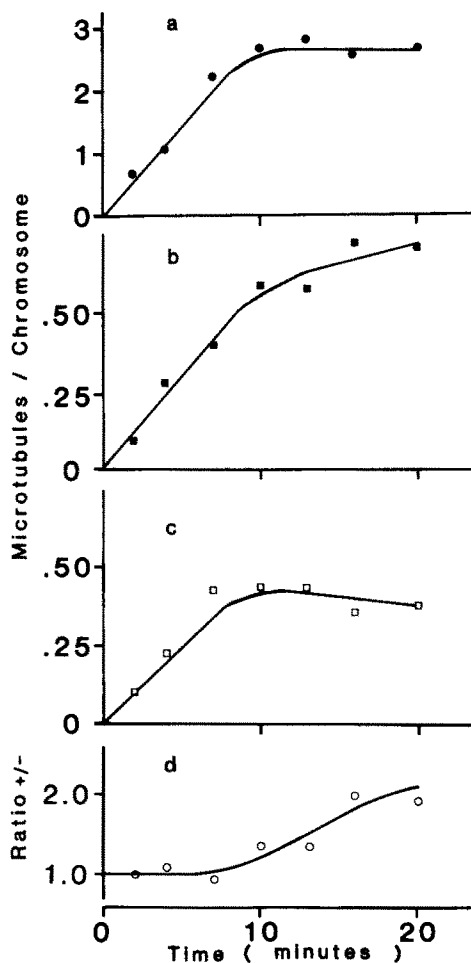
Figure 5. Kinetics of capture as a function of microtubule polarity. Stable segmented microtubules were incubated with chromosomes in the capture buffer containing 4 µM taxol at 37°C for various periods of time where aliquots were fixed, sedimented onto coverslips, and processed by immunofluorescence to visualize microtubules, their biotinylated segments and chromosomes. (*a*) Total (+ end, − end or ambiguous) microtubules captured by kinetochores (●); (*b*) microtubules captured by their + end (■); (*c*) microtubules captured by their − end (□); (*d*) ratio of + end- vs.− end-captured microtubules (○).
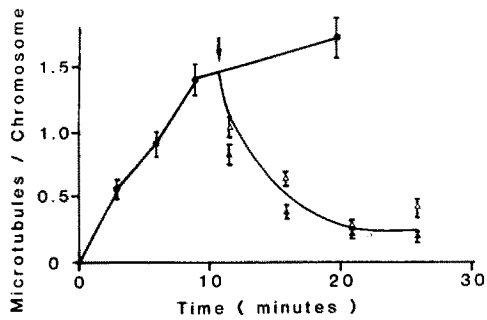
Figure 6. Capture of microtubules by kinetochores and their dissociation as a function of the nucleotide. Stable microtubules were incubated with chromosomes in our routine conditions including 4 μM taxol at 37°C for various periods of time (●). After 11 min, aliquots were diluted 10-fold in the same buffer containing again 4 μM taxol and 1 mM ATP-Mg (▲) or 1 mM UTP-Mg (△). Aliquots were taken all along the experiment, were fixed and analyzed by immunofluorescence after sedimentation on coverslips. Microtubules unambiguously bound to kinetochores were scored. At least 100 microtubules were analyzed per time point. Error bars indicate the SEM.

with a half time of ~5 min. The rate of dissociation is the same in the presence of UTP or ATP.

Effect of Microtubule Polarity on the Dissociation Rate of Captured Microtubules

To investigate dissociation rates as a function of the polarity of the captured microtubules we incubated chromosomes with polar seeds stabilized with taxol and after 10 min, diluted the mixture 10-fold. The dissociation rates for plus end or for minus end capture were determined separately by assessing the number and polarity of the remaining microtubules. As shown on Fig. 7 *a*, the overall capture reaction reaches a plateau and dissociation was rapid after dilution. As in the experiment in Fig. 5 the rates of binding of microtubule plus ends or minus ends were very similar in the first 10 min. However, after 10 min, an increasing number of microtubule plus ends were captured while minus ends were lost (see Figs. 5 and 7, *b* and *c*). After dilution (Fig. 7) microtubules captured at their minus ends were lost at a rate seven times greater ($t_{1/2}$ = 2.4 min) than those captured at their plus ends ($t_{1/2}$ = 17 min). Therefore the apparent higher affinity of kinetochores for microtubule plus ends as a function of time (Fig. 7 *a*) is due to similar rates of binding to both ends, coupled with a seven-fold higher rate of dissociation from the minus end.

Discussion

The attachment of chromosomes to the mitotic spindle takes place during prophase at a time of rapid chromosome movement and dynamic microtubule growth. Simplification of these processes in vitro is essential to understand the detailed mechanisms, yet it runs the risk of omitting important interactions. In this paper, we have tried to consider in vitro the initial interaction of microtubules and chromosomes in the absence of microtubule dynamics. To do this dynamics were suppressed with the drug taxol. We have shown that the capture reaction proceeds as efficiently with taxol-stabilized
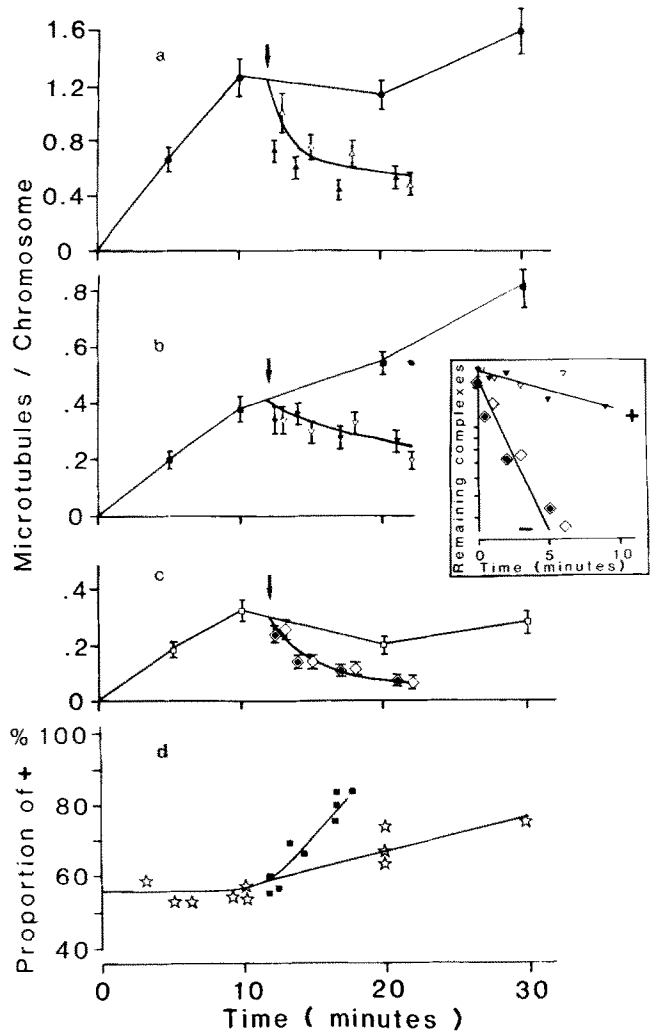
Figure 7. Capture and dissociation of microtubules from kinetochores as a function of their polarity. Stable segmented microtubules were incubated in the presence of chromosomes as usual in the presence of 4 μM taxol for various periods of time at 37°C where aliquots were fixed and processed for immunofluorescence (●, ■, and □ in *a*, *b*, and *c*, respectively). After 11 min of capture, part of the sample was diluted 10-fold in the same buffer containing 1 mM ATP-Mg (▲, ▼, ◆ in *a*, *b*, and *c*) or 1 mM UTP-Mg (△, ▽, ◇ in *a*, *b*, and *c*) and aliquots were analyzed as above. In *a*, the total number of microtubules captured by kinetochores is expressed as a number of microtubule per chromosome as a function of time. (*b*) The number of microtubules unambiguously captured by their + end. In *c*, the − end data is presented. The inset shows a semilogarithmic representation of the number of complexes (microtubules captured by chromosomes) remaining after dilution as a function of time. The figures are taken from *b* for the + end data, with (▼) or without (▽) 1 mM ATP-Mg, and from *c* for the − end datas, with (◆) or without (◇) 1 mM ATP-Mg. In *d*, we represented + end vs. + and − end (proportion of + end) captured microtubules as a function of time during the capture reaction (☆) or during the dissociation step (■). Several independent experiments were superimposed on the same graph. At least 100 microtubules were analyzed per time point. Error bars give the SEM.

microtubules as with dynamic microtubules. We also showed that treatment with taxol does not measurably affect the capacity of the ends of the microtubules for subsequent elongation. Yet not all the properties of taxol-stabilized

microtubules are known and taxol could affect some of the properties of kinetochore microtubule interactions that are not presently understood.

For convenience and accuracy in analysis, microtubules showing asymmetric growth of unmodified tubulin from biotin labeled seeds were used to determine the polarity of capture. As before, we found that the initial capture reaction reached a plateau within 10 min, and that the initial capture was equally likely for microtubules with either polarity. The number of microtubules at the plateau increased with increasing microtubule concentration under conditions where the number of microtubules in solution was not depleted by binding. After reaching the plateau the association was still dynamic. Dilution resulted in rapid loss of microtubules. Continued incubation resulted in a progressive replacement of microtubules captured at the minus end with those captured at the plus end.

Previous estimates of the rate of microtubule binding suggested that they might be diffusion controlled (Hill, 1985). We estimate from these experiments that the initial rate of microtubule addition for both plus and minus ends was 1 min^{-1} (0.017 s^{-1}) at a concentration of 7.2 $\times$ 10^{10} microtubules/ml. This gives a value of 1.2 $\times$ $10^8 s^{-1} M^{-1}$ which again is similar to diffusion control reaction rates. The half-life for microtubules captured at the minus end is 2.4 min which yields an off rate of 4.8 $\times$ $10^{-3} s^{-1}$ and the half life of those captured at the plus end is 17 min, which gives an off rate of 6.8 $\times$ $10^{-4} s^{-1}$. The dissociation constant for the minus end capture would be 3.8 $\times$ 10^{-11} M and for the plus end is 5.3 $\times$ 10^{-12} M. Given the concentration of microtubule ends in a mitotic cell (10^{15}–10^{16} per l or $\sim10^{-8}$ M) almost all of the sites on the kinetochore should be occupied and the turnover will be regulated by the dissociation process. The free energy of binding at 37°C for minus end capture would be 14.8 kcal/mole and for the plus end 16.0 kcal/mole. Thus plus end specificity is due to only an additional 1.2 kcal/mole. These values represent relatively small chemical forces.

We may make an estimate of whether this strength of interaction is sufficient to tether the chromosome during chromosome movement. A precise calculation would require knowledge of the exact profile of potential energy distribution versus distance (Hill and Kirschner, 1982). However, a minimum value of the strength of interaction could be calculated assuming that the energy is distributed over the width of the kinetochore which we can assume is 40 nm. This would give an energy vs. distance profile that started at zero and decreased linearly to a minimum at 40 nm, the extremity of the kinetochore plate. The potential energy curve would have a slope equal to the force and the product of the force and distance equal to the total energy, which for plus ends is 16.0 kcal or 67 kJ. The linear force under these assumptions would be 2.8 $\times$ 10^{-7} dynes. The force required to move chromosomes through an aqueous medium has been estimated at only 10^{-8} dynes, which would require a force per microtubule of only 2 $\times$ 10^{-10} dynes assuming 50 microtubules/chromosomes. However, Nicklas (1983) has measured the maximum force per chromosome in vivo at 7 $\times$ 10^{-5} dynes in grasshopper spermatocytes which would be 1.4 $\times$ 10^{-6} dynes per microtubule (assuming 50 microtubules in mammalian cells). Thus, the strength of interaction calculated from the equilibrium constant and assuming in the simple model of the potential well is only $\sim20\%$ of that needed to tether a chromosome against the measured sustaining forces in grasshopper spermatocytes. Given all of the assumptions this value for the strength of attachment is not far from the measured value of the force exerted. This may suggest that there are some phylogenetic differences or decrease in the strength of attachment produced by chromosome preparation. Alternatively there may be a different mode of microtubule growth, such as attachment deeper into the kinetochore that may be necessary for stable attachment. Most likely the potential energy profile may not be linear and the force would act over a shorter distance. Finally other components either of the microtubule or the kinetochore may be necessary for more stable attachment.

The similarity of the on rates for plus and minus ends is not surprising if the process is diffusion controlled. If the kinetochore associated with the microtubule laterally there should be no difference in the stability of the interaction, therefore even this relatively small (1.2 kcal) difference in stability is strong evidence that microtubule interaction senses the polarity of the microtubule, which it can only if the interaction is near the ends. We may also conclude that the small difference in the energy of interaction suggests that most of the interaction is similar at the plus and minus ends. The most plausible model for these parameters is that the kinetochore interacts laterally but near the ends of the microtubule.

The importance of these specific rates and affinities may be in the morphogenesis of the spindle itself. Microtubules can attach readily to chromosomes by random diffusion. Microtubules which are probably all initiated at the centrosome have the proper polarity (plus end distal) and once an interaction is made it is relatively stable ($t_{1/2}$ = 17 min). Attachment of a kinetochore to one pole would constrain the geometry of microtubules from the other pole so they could only interact at the kinetochore at an oblique angle. Such oblique lateral interactions may be of similar stability to the interactions between the kinetochores and the minus end of microtubules. The lateral component would be similar but the end component could be lacking. Such microtubules would dissociate more rapidly, leading to the establishment of a proper metaphase state.

We have found that the interaction of taxol-stabilized microtubules with the kinetochore is not affected by ATP. We saw no effect on the rate of association or dissociation of either the plus or minus ends. In the initial interaction we saw no change in the fraction of biotin segments distal to the kinetochore, nor the distance between the kinetochore and the biotin segment (our unpublished observations). These results suggest that in the presence of ATP no translocation had taken place. The failure of the seeds to translocate is not due to inhibition by taxol. Mitchison and Kirschner (1985) and Huitorel (unpublished observations) showed that translocation could take place in the presence of taxol if microtubules were elongated after capture and before taxol addition.

The experiments of Mitchison and Kirschner (1985b) showed that laterally attached chromosomes, produced by elongation of captured microtubules in the absence of ATP, could translocate to the plus ends. It left open the question of whether microtubules captured at the minus end could translocate to the plus end. The lack of any ATP effect when taxol-stabilized seeds are captured leads to the following
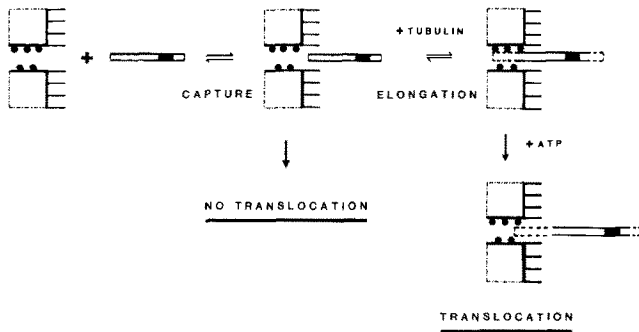
Figure 8. Model of kinetochore capture and translocation. (■) Biotinylated microtubule segment; (▢) unlabeled microtubule segment; (▮) portion of the kinetochore plate; (Ɛ) fibrous corona; (●) ATP dependent translocator.

conclusion. Microtubules captured at their minus ends cannot translocate. Either microtubules cannot be captured laterally in the center of the microtubule or, if they can, they cannot translocate. Microtubules captured at their plus ends could not be observed to translocate, since they were already at their plus ends. Interestingly such microtubules do not fall off at an increased rate as was reported for dynamic microtubules. Translocation has only been observed if microtubule growth occurs after the initial capture reaction. This result suggests that there are two types of interaction: an initial one that can occur in the absence of microtubule growth and a subsequent one that depends on elongation of the microtubule deeper into the kinetochore structure to allow for ATP dependent translocation (Fig. 8).

It must be remembered that the use of toxol to suppress microtubule dynamics, though affording a significant simplification, may alter the relevant mechanism of attachment. Though we have detected no affects of taxol other than to suppress microtubule assembly and disassembly, the dynamics themselves may be very important to the stability of the interactions. The experiments reported here say something very important about the kind of microtubule receptor or interaction sites that exist in the kinetochore. The binding is weak, perhaps just strong enough to allow the tethering of the chromosome during anaphase. Such weak interactions could allow the chromosome to make or break attachments that are unfavorable. The geometry of the kinetochore must allow it to distinguish the polarity of the microtubules and may be important in leading to the formation of a stable metaphase state.

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