

# Generation of Microtubule Stability Subclasses by Microtubule-associated Proteins: Implications for the Microtubule "Dynamic Instability" Model

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**ABSTRACT** We have developed a method to distinguish microtubule associated protein (MAP)-containing regions from MAP-free regions within a microtubule, or within microtubule subpopulations. In this method, we measure the MAP-dependent stabilization of microtubule regions to dilution-induced disassembly of the polymer. The appropriate microtubule regions are identified by assembly in the presence of [<sup>3</sup>H]GTP, and assayed by filter trapping and quantitation of microtubule regions that contain label. We find that MAPs bind very rapidly to polymer binding sites and that they do not exchange from these sites measurably once bound. Also, very low concentrations of MAPs yield measurable stabilization of local microtubule regions. Unlike the stable tubule only polypeptide (STOP) proteins, MAPs do not exhibit any sliding behavior under our assay conditions. These results predict the presence of different stability subclasses of microtubules when MAPs are present in less than saturating amounts. The data can readily account for the observed "dynamic instability" of microtubules through unequal MAP distributions. Further, we report that MAP dependent stabilization is quantitatively reversed by MAP phosphorylation, but that calmodulin, in large excess, has no specific influence on MAP protein activity when MAPs are on microtubules.

To perform their functions, microtubules form into characteristic intracellular arrays and structures. Within the same cell, different microtubule populations may exhibit a wide range of stability states which appear to reflect their metabolic activities (for review see reference 1). We have been studying a microtubule-associated protein (MAP)<sup>1</sup>, designated STOP (stable tubule only polypeptide), which has the capacity to form a highly stable subclass of microtubules. Such stable polymers are widely distributed in neuronal tissue (2) and are also characteristically represented in the kinetochore-to-pole

fibers of the mitotic spindle (3). There are also other, more subtle degrees of stabilization among microtubule populations. We have considered the possibility that unequal distributions among microtubules of the MAPs long known to be associated with them could cause such stability class distributions.

We have learned, from our studies on STOP protein, that exceedingly small amounts of STOP protein are profoundly effective in altering a microtubule's behavior (4). This characteristic is due to the fact that disassembly of the polymer is strictly end-wise (5-9), so that perhaps no more than one blocking molecule per end should be necessary to totally prevent disassembly. The MAPs commonly associated with recycled brain microtubules do not induce cold stability but do stabilize the microtubule, as we show here, against more subtle challenges such as dilution-induced disassembly. MAPs can substantially alter the ability of the polymer to assemble and to maintain a particular equilibrium state in vitro (10-13), and, due to the intrinsic disassembly characteristics of

<sup>1</sup> *Abbreviations used in this paper:* DG-MME buffer, 100 mM 2-(*N*-morpholino)ethanesulfonic acid, 1 mM MgCl<sub>2</sub>, 1 mM EGTA, 0.02% NaN<sub>3</sub>, pH 6.75, plus 10% glycerol, 5% dimethyl sulfoxide, and 16 mM MgCl<sub>2</sub>; MAPs, microtubule-associated proteins; MAP-microtubule protein, tubulin solution containing MAP proteins; MME buffer, DG-MME buffer without the glycerol, or dimethyl sulfoxide, but containing 1 mM MgCl<sub>2</sub>; PC-tubulin, pure tubulin obtained by phosphocellulose column chromatography; PLN, podophyllotoxin; STOP, stable tubule only polypeptide.

the polymer, they can act to stabilize polymers when present in very low titers.

We report here that MAPs have properties that can account for the formation of different stability classes of microtubules both in vivo and in vitro. These properties are (a) their ability to stabilize microtubules at very low ratios to tubulin, (b) their inability to either "slide" on one microtubule or exchange between microtubules and, (c) their extremely rapid, irreversible binding to polymers which can lead to an unequal distribution of MAPs at low MAP concentrations and therefore to a variety of stability classes between different polymers in the same solution. We also find that calcium, in the sub-millimolar range, and ATP reverse the observed MAP-dependent stabilization of microtubules. In our assays, calmodulin has no specific effect.

These results predict that MAPs at low concentrations can account for some of the observed stability classes of microtubules in the cell. Further, the generation of different stability classes in vitro can readily account for the paradoxical elongation of a subclass of microtubules during dilution-induced disassembly ("dynamic instability"), as recently reported (14).

## MATERIALS AND METHODS

**Materials:** All chemicals unless otherwise indicated were purchased from Sigma Chemical Co. (St. Louis, MO). [ $^3\text{H}$ ]GTP (25–50 Ci/mmol) was obtained from New England Nuclear (Boston, MA); nucleotides and acetate kinase were products of Boehringer Mannheim Biochemicals (Indianapolis, IN); podophyllotoxin (PLN), purified from the crude form supplied by Aldrich Chemical Co. (Milwaukee, WI), was the generous gift of L. Wilson. To perform the filtration assay, we used GF/C glass fiber filters from Whatman Inc. (Clifton, NJ). The buffer used throughout for protein purification, designated MME, contained 100 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), 1.0 mM  $\text{MgCl}_2$ , 1.0 mM EGTA, and 0.02%  $\text{NaN}_3$ , pH 6.75. For assay, MME buffer was modified as indicated below. Calmodulin was purified from beef brain by the methods of Watterson et al. (15), and the catalytic subunit of cAMP-dependent protein kinase type II was prepared from pig heart according to the protocol of Peters et al. (16).

**Microtubule Protein Isolation:** Cold labile microtubule protein from beef brain was isolated by three cycles of assembly and disassembly, in MME buffer, according to published procedures (17, 18), with the following modification. For the third assembly cycle, protein was resuspended from pelleted microtubules and centrifuged in MME buffer for 30 min, 120,000 *g* (average), at 4°C. The supernatant fraction was reassembled in 2 mM GTP at 30°C for 45 min, layered on 50% sucrose in MME buffer, and centrifuged for 2 h in a fixed-angle rotor (120,000 *g* average, 30°C). The pellets (yielding a tubulin solution containing MAP proteins, referred to as "MAP-microtubule protein") were stored at -80°C for later use. MAPs, when used in purified form, were isolated from MAP-microtubule protein by the methods of Sobue et al. (19) prior to use. Pure tubulin (PC-tubulin) was isolated from thrice-cycled purified microtubule protein by phosphocellulose column chromatography, from which pure tubulin elutes in the flow-through fraction (20). It is important to note that ATP has not been added to the preparation at any stage during purification.

**Assays:** The microtubule assembly state was monitored by turbidity measurement as described (21). The filter assay, to determine radioactive regions of microtubules labeled with [ $^3\text{H}$ ]GTP, was performed essentially as previously described (22, 23). Protein for the assay (either MAP-microtubule protein or PC-tubulin or a mixture of the two) was assembled at 30°C in MME buffer plus 10% glycerol (vol/vol), 5% dimethyl sulfoxide (vol/vol), and 16 mM  $\text{MgCl}_2$  (designated DG-MME buffer). The buffer also contained 0.05 mM GTP, 10 mM acetyl phosphate, 0.1  $\mu\text{g}/\text{ml}$  acetate kinase, and, when appropriate, [ $^3\text{H}$ ]GTP at 20  $\mu\text{Ci}/\text{ml}$ . Protein concentration was 1.5 mg/ml. Assembly was routinely performed by incubation for 50 min at 30°C, shearing, and additional incubation for 15 min prior to any protein mixture or other experimental manipulations. At time points reactions were terminated by 25-fold dilution of protein samples in MME buffer containing 50% sucrose, 1% glutaraldehyde, and 25  $\mu\text{M}$  PLN. These conditions quantitatively maintained microtubules in the assembled state representative of the time point, as determined in Fig. 1. Samples were then applied to glass fiber filters and assayed for radioactivity. Shearing, when performed, was done by passage of microtubule

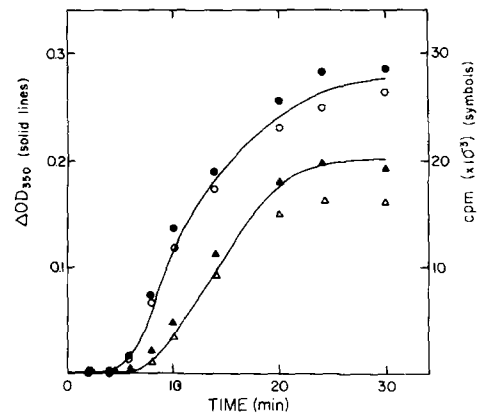


FIGURE 1 Validation of the [ $^3\text{H}$ ]GTP filter binding assay. MAP-microtubule protein and PC-tubulin (both at 1.5 mg/ml protein) were assembled at 30°C in DG-MME buffer containing [ $^3\text{H}$ ]GTP. The assembly of both solutions was monitored by turbidity change at 350 nm as shown. Assembly of MAP-microtubule protein is represented by the upper curve; assembly of PC-tubulin is represented by the lower curve. At the indicated time points, 40  $\mu\text{l}$  of each incubation mixture was pipetted directly into 1 ml of 50% sucrose-MME buffer and subsequently filter assayed. ●, MAP-microtubule protein diluted into 50% sucrose buffer containing 1% glutaraldehyde. ○, MAP-microtubules diluted into buffer containing no glutaraldehyde. ▲, PC-tubulin microtubules diluted into 50% sucrose buffer containing 1% glutaraldehyde. △, PC-tubulin microtubules diluted into buffer containing MAP-microtubule protein, at 6% final MAP concentration. All 50% sucrose-MME dilution buffers contained 25  $\mu\text{M}$  PLN.

protein through a 25-g needle three times. All results have been generated from duplicate points.

In a typical experiment, the fully assembled state of either PC-tubulin or MAP-microtubule protein yielded 30,000 to 40,000 cpm per 40  $\mu\text{l}$  sample applied to a filter (with an SD averaging  $\pm 3\%$  of the final cpm). 1 ml contains 50 nmol of GTP total (GTP = 50  $\mu\text{M}$ ), and 20  $\mu\text{Ci}$  (or  $\sim 2 \times 10^7$  cpm); or  $4 \times 10^5$  cpm/nmol. Therefore, 1.87–2.49 nmol of GTP, of the 50 nmol present per ml, have been incorporated into microtubules and retained on filters. For the case of pure tubulin, present at 1.5 mg/ml,  $\sim 40\%$  assembles into polymer (based on sedimentation assay); this is equivalent to 0.6 mg/ml or 6.0 nmol/ml (based on a molecular mass of 100,000 daltons). Assuming  $\sim 50\%$  molar incorporation of [ $^3\text{H}$ ]GTP into microtubules (7) and that GF/C filters trap microtubules with 70% efficiency (22), we would predict that 2.25 nmol/ml of GTP would bind to filters in an assay. This figure lies in the middle of the range actually found (1.87–2.49 nmol) in our assays.

By length measurement from electron micrographs of microtubules, we have found that MAP-microtubules (6% MAPs) in a typical experiment have an average length of 5.0  $\mu\text{m}$ ; PC-tubulin microtubules, 5.9  $\mu\text{m}$ ; microtubules formed with PC-tubulin "tails" on MAP-microtubule "seeds," 8.3  $\mu\text{m}$ . At high magnification, all microtubules, including those in glutaraldehyde STOP buffer, were morphologically normal.

## RESULTS

### Validation of the [ $^3\text{H}$ ]GTP Filter Binding Assay

As described in Materials and Methods, we use a filter binding assay to quantitate the microtubule assembly and stability state. We and others have extensively validated the quantitative nature of the basic assay. For the purposes of this study, the assay has been modified by the use of 1% glutaraldehyde to trap microtubules in an assembly state indicative of a time point. This modification was necessitated by the extraordinary lability of PC-microtubules (as shown in Fig. 2), for which we can obtain reliable time points only by the addition of glutaraldehyde.

Fig. 1 shows a comparison of microtubule assembly, as measured by turbidity, with assembly as measured by [ $^3\text{H}$ ]-

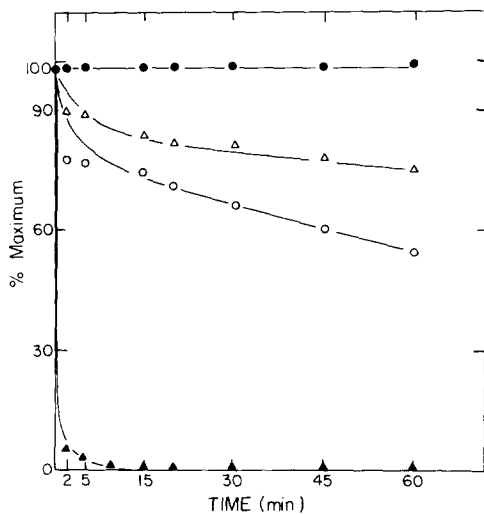


FIGURE 2 Stability of microtubules after dilution into sucrose-containing buffers. MAP-microtubule protein or PC-tubulin was assembled at 30°C for 50 min in DG-MME buffer containing [<sup>3</sup>H]GTP. Microtubules were sheared, incubated an additional 15 min, then diluted in 30°C MME containing either 50% or 30% sucrose and 25 μM PLN. At the indicated time points, 1-ml aliquots were removed and mixed with 25-fold stock glutaraldehyde to a final concentration of 1% glutaraldehyde. Points at time zero were determined by pipetting 40 μl of the original incubation mixture directly into 1 ml of 50% sucrose which contained 1% glutaraldehyde. Results are expressed as percent of the time zero assembly level. Radioactivity of the 100% value was ~32,000 cpm per 40-μl aliquot for MAP-microtubule protein and 26,000 cpm for PC-tubulin; blanks (no assembly) were ~600 cpm. ●, MAP-microtubule protein diluted into 50% sucrose; ○, MAP-microtubule protein into 30% sucrose; △, PC-tubulin into 50% sucrose; ▲, PC-tubulin into 30% sucrose.

GTP label incorporation. For MAP-microtubule protein (Fig. 1), label incorporation parallels assembly with high reliability. As these microtubules are completely stable in 50% sucrose buffer, they may be assayed without the addition of glutaraldehyde. However, it can be seen that the presence of 1% glutaraldehyde in the 50% sucrose STOP buffer does not substantially alter the assay result (Fig. 1, compare closed circles and open circles). PC-microtubules must be stabilized for assay at time points by addition of glutaraldehyde, yet it is clear that, here too, label incorporation parallels the assembly state measured by turbidity (Fig. 1). The ratio of label incorporation to  $\Delta$  OD is the same for both MAP-microtubules and PC-microtubules, blocked with glutaraldehyde. Addition of MAPs at a time point to stabilize PC-tubulin microtubules is also quite effective, although there is a reproducible loss of label due to subunit dissociation during the course of MAP binding. For this reason, glutaraldehyde blockage was chosen for use as an end point determinant in the following experiments.

This experiment demonstrates that the filter assay is a reliable measure of the microtubule-assembled state, regardless of the presence or absence of glutaraldehyde or the presence or absence of MAPs. This concentration of glutaraldehyde can be used for assay with equivalent results in 30% sucrose-MME buffer, as well as in DG-MME buffer.

#### Quantitation of MAPs on Microtubules

To determine the distribution of MAP proteins among

microtubule subpopulations, one must have an assay that clearly distinguishes between MAP-containing and MAP-free microtubule regions. We have developed an assay which is able to detect MAP proteins in very low concentrations on microtubules. This assay depends on the capacity of MAPs to substantially protect microtubules from dilution-dependent disassembly in sucrose-containing buffers.

When fully assembled MAP-saturated microtubules, labeled with [<sup>3</sup>H]GTP, are diluted 25-fold into a buffer containing 50% sucrose, they are indefinitely and completely stable (Fig. 2) (see also reference 7); when diluted in a 30% sucrose buffer they slowly disassemble, reaching 50% of their original state in 1 h (Fig. 2). PC-tubulin is strikingly less stable to these two dilution conditions. Microtubules assembled from PC-tubulin slowly dissociate in 50% sucrose, but are almost totally disassembled by the first time point in 30% sucrose (Fig. 2). A comparison of the 10-min time points in 30% sucrose for MAP-saturated and MAP-free microtubules shows this time point can be used to strongly distinguish between the two cases. MAP-containing microtubules have 20% disassembled while the MAP-free polymers have totally disassembled. Close inspection of the data in Fig. 2 shows that the dilution response is apparently biphasic. The reason for this behavior is unknown.

We have used these data to establish an assay in which we determine the relative stability of microtubules due to MAP content. The assay involves taking the ratio of surviving microtubules after 10 min of dilution into 30% sucrose versus the full assembly state in 50% sucrose. We find this disassembly assay to be quite sensitive to the presence of low levels of MAPs.

Using this assay, we have generated a dose-response curve for the effect of MAPs on the stabilization of microtubules against dilution. Recycled MAP-microtubule protein was mixed in increasing ratio (but at constant protein concentration) with pure tubulin. The mixtures were then assembled in the presence of [<sup>3</sup>H]GTP. At steady state the samples were diluted into 30% sucrose buffer for 10 min, then trapped on glass fiber filters and compared with the original assembly state for their stability ratio. The result (Fig. 3) shows that a stabilization of microtubules is evident at very low MAP concentration (~1% of the total protein). Maximal stability of the microtubule population is attained at high MAP ratios which possibly represent saturation of the MAP binding sites on microtubules. To create MAP-stabilized microtubules for MAP distribution studies, we use a MAP-to-tubulin ratio of 6% (the percentage attained at a MAP-microtubule protein PC-tubulin ratio of 0.3:1.0 on the curve in Fig. 3), well below the saturation level.

Microtubule stabilization, as measured here, is induced by a mixed MAP population. To determine which MAP species are responsible for protection against dilution-induced disassembly, we have measured the stabilization induced by phosphocellulose column-purified MAP fractions, separated by column chromatography on an aCa 22 Ultragel (IBF) column in MME buffer plus 0.1 M NaCl. We found two peaks of high specific activity, one associated with MAP-2 and the other with tau (Pirrollet, F., D. Job, and R. Margolis, unpublished results). The specific activities of the two peaks showed that MAP-2 was ~6.7 times more efficient at stabilizing microtubules than tau, on a molar basis. There was also a low specific activity peak associated with the void fraction, which apparently contained MAP-2 and tau aggregates.

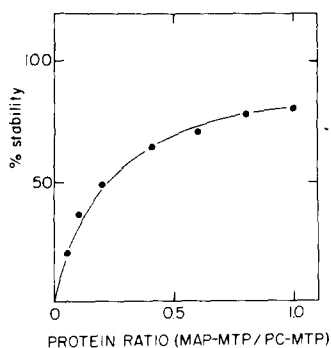


FIGURE 3 Dose-response curve of MAP protection of microtubules upon dilution. MAP-microtubule protein was mixed in increasing ratio with PC-tubulin, to the proportions indicated (protein ratio = MAP-microtubule protein/PC-tubulin). The mixtures were then incubated for 50 min at 30°C in DG-MME buffer, then sheared and incubated an additional 15 min. At the end point, an aliquot of 40  $\mu$ l was diluted into 1 ml of 50% sucrose containing 1% glutaraldehyde, and another 40- $\mu$ l aliquot was diluted into 30% sucrose, incubated 10 min, then brought to 1% glutaraldehyde. For each protein ratio, results are expressed as percent of stability (30% sucrose vs. 50% sucrose counts) by filter assay. The 50% sucrose values were 33,000 cpm (1:1), 40,000 cpm (0.8:1), 37,000 cpm (0.6:1), 34,000 cpm (0.4:1), 28,000 cpm (0.2:1), and 29,000 cpm (0:1), and unassembled blanks 600 cpm. In the assembly incubation protein concentrations were always 1.5 mg/ml. MAP-microtubule protein contained ~25% MAPs as determined by scans of stained gels. Therefore the mixture 0.3:1 contains ~6% MAPs.

### Measurement of Exchange of MAPs Between Microtubules

Having established a sensitive assay for the presence of MAPs on microtubules, we designed experiments to test for the exchange of MAPs between microtubules. PC-microtubules were assembled in the presence of [<sup>3</sup>H]GTP, then mixed in a 1:1 ratio with either assembled or disassembled MAP-containing microtubule protein. The mixture experiment was performed after 25-fold dilution in 50% sucrose buffer + PLN, to prevent MAP-microtubule assembly. Upon mixture with unassembled MAP-microtubule protein, the labeled microtubules are rapidly stabilized (Fig. 4A). Stabilization is essentially complete after 5 min, even at this high dilution of protein. On the contrary, mixture with preassembled MAP-microtubules yields no detectable stabilization of the labeled microtubules, even after 2.5 h (Fig. 4A). We conclude that MAPs bind rapidly to microtubules, and do not measurably exchange off them once bound. Further, tubulin subunits do not measurably compete with microtubules for MAP binding.

In a reverse experiment, we labeled MAP-microtubules, then mixed them upon dilution with either subunits or assembled microtubules composed of pure tubulin. The presence of tubulin, either in an assembled or unassembled state, had no competitive influence on MAPs bound to the labeled microtubules (Fig. 4B). The degree of stability did not change over 2.5 h and was the same as that observed in the absence of added protein.

### Assay for Migration of MAPs on Microtubules

Since [<sup>3</sup>H]GTP incorporation into microtubule polymers serves as a position marker for the labeled tubulin subunit, one can use hybrid polymers, labeled in specific regions, to assay the migration of stabilizing proteins from one region of

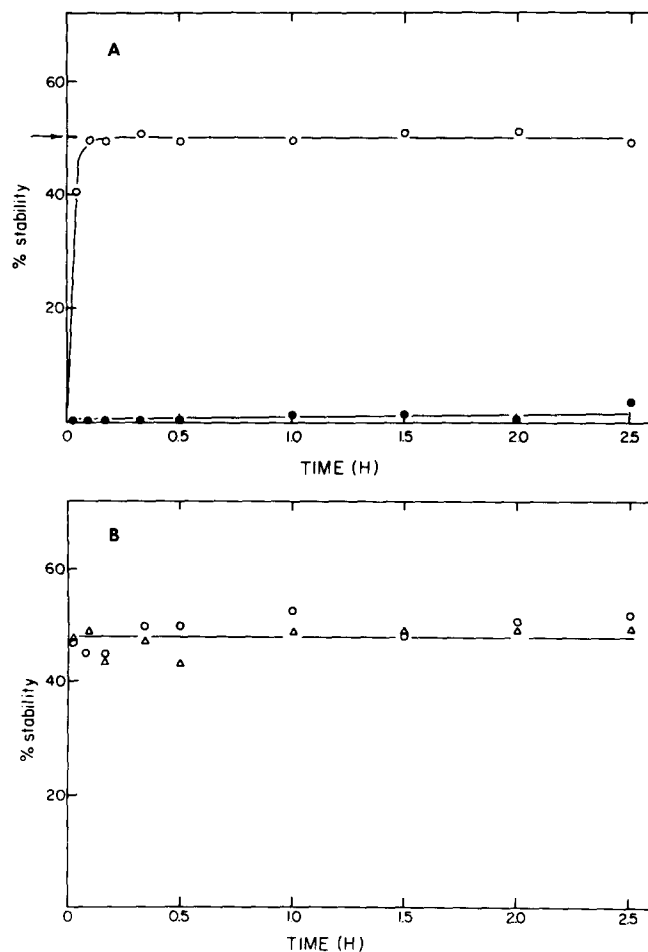


FIGURE 4 Assay of exchange of MAPs between microtubules. (A) Exchange of MAPs onto PC-tubulin microtubules. PC-tubulin (1.5 mg/ml) was assembled under standard conditions, with [<sup>3</sup>H]GTP. After assembly, the protein was diluted 25-fold in 50% sucrose-MME with 25  $\mu$ M PLN. At time zero, this protein was mixed with an equal volume and concentration of either assembled or disassembled MAP-microtubule protein (6% MAPs), which was in a similar state of 25-fold dilution. At time points the stability of the [<sup>3</sup>H]-labeled PC-tubulin microtubules was assayed by removing two 1-ml aliquots, one of which was brought to 1% glutaraldehyde. The other aliquot was mixed with MME to yield a 30% sucrose solution, and was brought to 1% glutaraldehyde after 10 min. Results (percent stability) represent the ratio of counts in the 30% sucrose vs. the 50% sucrose samples. O, stability level of PC-tubulin microtubules mixed with subunits; ●, stability of PC-tubulin mixed with pre-assembled MAP-microtubules. The arrow on the Y axis indicates the stability of MAP-microtubules alone (containing 6% MAPs). (B) Exchange of MAPs from MAP-microtubules. In this reverse experiment, the protocol was identical to that above, except that the MAP-microtubules (6% MAPs) were labeled by assembly with [<sup>3</sup>H]GTP, then mixed at steady state with unlabeled PC-tubulin, either in assembled or unassembled state. O, stability level of MAP-microtubules mixed with PC-tubulin subunits; Δ, stability level of MAP-microtubules mixed with pre-assembled PC-tubulin microtubules.

the polymer to another. Recently we have used this strategy to demonstrate that the STOP protein exhibits "sliding" behavior on the polymer (23). As with the STOP protein, MAPs do not exchange from one polymer to another. Any displacement relative to tubulin subunits can therefore be interpreted as sliding.

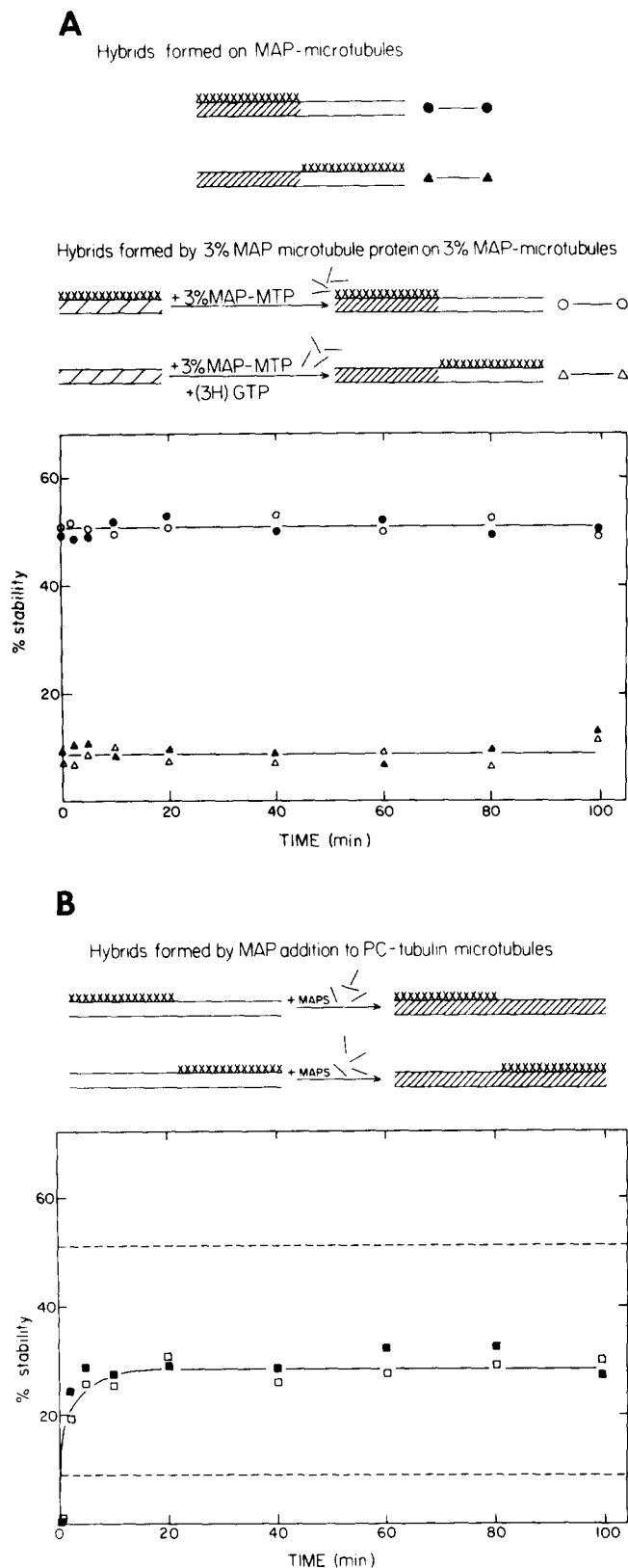


FIGURE 5 Assay for migration of MAPs on microtubules. (A) Assay of microtubule hybrids with initial asymmetric MAP distribution. MAP-microtubule protein (6% MAPs; 1.5 mg/ml) was assembled under standard conditions (50 min assembly, shearing, 15 min further equilibration) in DG-MME buffer, either in the presence or absence of  $[^3\text{H}]\text{GTP}$ . These microtubules were then mixed in 1:1 ratio with PC-tubulin. Labeled MAP-microtubules were mixed with unlabeled PC-tubulin in a 20-fold GTP chase, while unlabeled

Hybrid microtubules were constructed by assembling MAP-microtubules with  $[^3\text{H}]\text{GTP}$ , shearing, then incubating with pre-warmed pure tubulin in the presence of a GTP chase for 10 min. The pure tubulin will not self-assemble within the first 10 min under these conditions and is constrained to assemble as "tails" on the pre-existing MAP-microtubule "seeds" (see Fig. 7, which shows that PC-tubulin does not initiate assembly within 10 min under these conditions). MAPs cannot add to these tails since equilibrium dissociation of MAPs from MAP-microtubules does not measurably occur (Fig. 4). The hybrid microtubules are then diluted in 50% sucrose to prevent further assembly and assayed at time points for destabilization of labeled MAP regions, or for stabilization of pure tubulin tails. The result (Fig. 5A) shows clearly that there is no stabilization of MAP-free regions with time, and no destabilization of MAP-containing regions. The low level of stability on MAP-free regions remains constant and is probably due to small amounts of MAP displacement due to temperature fluctuation during mixing and dilution. When care is taken to maintain pipette tips and receiving solutions at  $30^\circ\text{C}$ , this background is minimized. This experiment was also performed by an alternate method, with identical results.

MAP-microtubules were mixed with PC-tubulin with the simultaneous addition of  $[^3\text{H}]\text{GTP}$ . The mixture of MAP-microtubule "seeds" and PC-tubulin subunits was incubated 10 min to form PC-tubulin tails, then diluted 25-fold in 50% sucrose-MME to prevent further assembly or disassembly during the migration assay. At the indicated time points, 1 ml of each solution was brought to 1% glutaraldehyde; while another ml was diluted with MME to 30% sucrose, left 10 min at  $30^\circ\text{C}$ , and then brought to 1% glutaraldehyde. Results, expressed as percent stability, represent the ratio of 30% sucrose vs. 50% sucrose counts. The composition of the hybrids generated is depicted in the figure: XXX indicates a labeled region of the polymer, and //// indicates a MAP-containing region. ●, labeled MAP-microtubule seeds and unlabeled tails; ▲, unlabeled MAP-microtubule seeds and labeled tails. Alternatively, microtubule hybrids with asymmetric MAP distribution were generated by assembling MAP-microtubule protein containing 3% MAPs (conditions identical to MAP-microtubule protein assembly as indicated above) either with or without  $[^3\text{H}]\text{GTP}$ , then adding 3% MAP-microtubule protein in 1:1 ratio either with a 20-fold GTP chase or with  $[^3\text{H}]\text{GTP}$ . After 10 min, the samples were diluted 25-fold into 50% sucrose-MME and time points were taken and processed as indicated above. The composition of the hybrids generated in this manner is depicted in the figure, with the same symbols as above. ○, labeled MAP-microtubule seeds and unlabeled tails; △, unlabeled MAP-microtubule seeds and labeled tails. (B) Assay of microtubule hybrids with initial symmetric MAP distribution. PC-tubulin (1.5 mg/ml) was assembled under standard conditions (as above for MAP-microtubule protein) either in the presence or absence of  $[^3\text{H}]\text{GTP}$ . These microtubules were then mixed in 1:1 ratio with PC-tubulin. Labeled microtubules were mixed with unlabeled PC-tubulin in a 20-fold GTP chase, while unlabeled microtubules were mixed with PC-tubulin with the simultaneous addition of  $[^3\text{H}]\text{GTP}$ . The mixtures were then incubated 10 min to form PC-tubulin tails. These microtubules were then diluted 25-fold in 50% sucrose-MME and mixed with purified MAPs to a final 6% MAP-to-tubulin ratio to distribute MAPs evenly between labeled and unlabeled microtubule regions. Time points were then removed and processed as indicated above. The composition of the hybrids generated is depicted in the figure: XXXX indicates a labeled region of the polymer, and //// indicates a MAP-containing region. ■, labeled seeds and unlabeled tails; □, unlabeled seeds and labeled tails. The dashed lines indicated the stability distributions obtained with asymmetric hybrids in A.

MAP-microtubules containing half the usual MAP concentration (3%) were assembled either with or without label. These microtubules were then incubated with 3% MAP-microtubule protein to form labeled "tails" on unlabeled polymer seeds or unlabeled "tails" on labeled polymer seeds. The asymmetry in stability regions (Fig. 5A) was identical to the result above in which MAP-containing and MAP-free tubulin were used to construct hybrids. This result indicates to us that MAPs added with subunits during "tail" formation bind to pre-existing microtubules at a rate much more rapid than the rate of tubulin subunit assembly.

The observed asymmetry in disassembly can be due to absence of MAPs from pure tubulin tails or to an intrinsic asymmetry in disassembly of these polymers, on which MAPs have slid and redistributed evenly by the first time point. To discount the latter possibility, we have performed a control experiment, in which PC-microtubules were constructed with [<sup>3</sup>H]GTP label either in the polymeric seeds or in the "tails" (Fig. 5B). After attaining steady state, these hybrid microtubules were diluted in 50% sucrose and mixed with MAPs to the same final ratio as the hybrids in Fig. 5A. The difference was that, in this case, the MAPs were purposefully distributed evenly on the microtubules. Time points to determine stability of the different regions showed that each labeled portion of the polymer was equally stable (Fig. 5B). Differential disassembly of microtubule ends after MAP redistribution, therefore, cannot account for the constant asymmetry of the result in Fig. 5A. The alternate possibility is therefore confirmed, and we conclude that MAPs do not measurably slide on microtubules under these conditions.

#### Assay of Local MAP Stabilization in Steady-State Microtubules

One advantage of the assay system used for the experiments thus far presented is that it allows for the study of MAP redistribution on microtubules in the absence of tubulin subunit flux, thus avoiding the experimental complications that tubulin redistribution might cause. One weakness of the 50% sucrose-MME buffer system is that it could conceivably stabilize MAP-tubulin interactions, in the same manner that it stabilizes tubulin-tubulin interactions within the polymer.

Having established that MAPs do not exchange between microtubules, or redistribute upon a single polymer in 50% sucrose-MME buffer, we wished to determine the behavior of MAPs on steady-state microtubules. We therefore repeated the experiments on "hybrid" microtubules, as presented in Fig. 5, but with all assembly and all assay time points being performed in DG-MME buffer. The result shows that MAP-free tails form rapidly and maintain a steady state through the course of the experiment, while MAP-containing "heads," labeled specifically, lose their label slowly and linearly to an apparent treadmilling reaction (Fig. 6A). The rate of subunit loss from the head region is ~12% per h.

It is clear that there is no rapid redistribution of MAPs under these steady-state conditions. The percent stability of the original MAP-containing "head" region does not change (Fig. 6B), indicating that MAPs are not measurably lost from the residual head region in the course of this experiment. There is a slow and linear gain in stability of the labile "tail" regions (Fig. 6B) which can be fully accounted for by the loss of MAPs with tubulin from treadmilling head regions, and the subsequent redistribution of these MAPs.

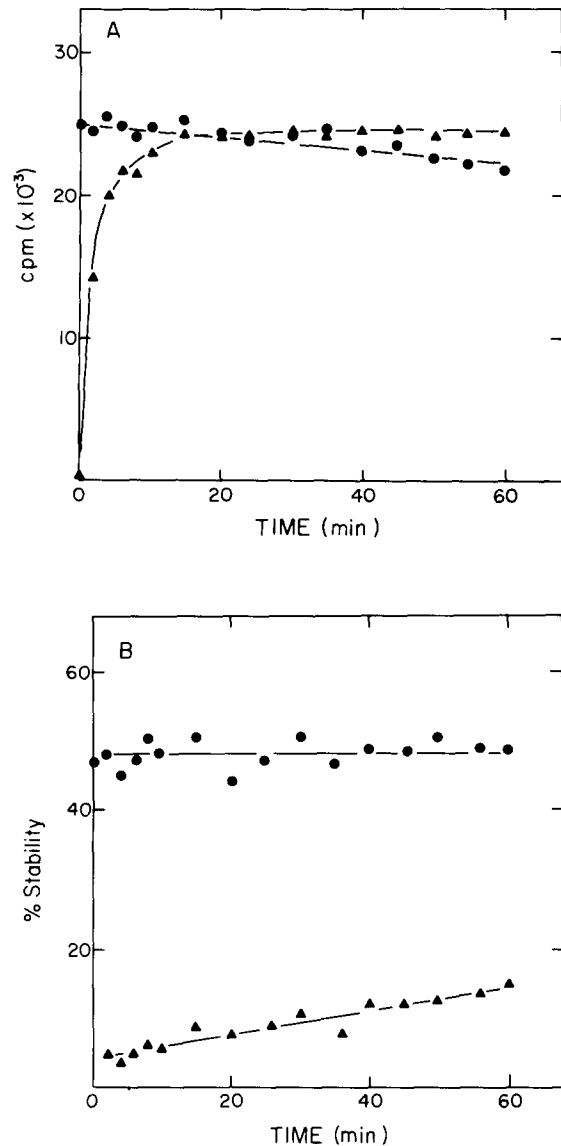


FIGURE 6 Assay of MAP stabilization in steady-state microtubules. Microtubule hybrids with initial asymmetric MAP distribution were formed with the same experimental protocol as in Fig. 5, except that MAP-microtubule "seeds" were not sheared prior to mixture with PC-tubulin. MAP-microtubule protein (6% MAPs; 1.5 mg/ml) was assembled for 50 min in DG-MME buffer either in the presence or absence of [<sup>3</sup>H]GTP. These microtubules were then mixed in 1:1 ratio with PC-tubulin. Labeled MAP-microtubules were mixed with unlabeled PC-tubulin in a 20-fold GTP chase, while unlabeled MAP-microtubules were mixed with PC-tubulin with the simultaneous addition of [<sup>3</sup>H]GTP. All samples were then maintained in DG-MME buffer until the time points indicated. (A) Assay of the assembly state of microtubule "head" and "tail" regions. At the indicated time points (time zero being the time of addition of PC-tubulin) 40  $\mu$ l of each sample was diluted into 1 ml of 50% sucrose-MME buffer containing 1% glutaraldehyde and 25  $\mu$ M PLN. ●, labeled MAP-microtubule seeds and unlabeled tails; ▲, unlabeled MAP-microtubule seeds and labeled tails. (B) Assay of MAP stabilization of the different microtubule regions. At the indicated time points, time zero being the time of addition of PC-tubulin, 40- $\mu$ l aliquots were diluted into 1 ml of 30% sucrose-MME, left 10 min at 30°C, and then brought to 1% glutaraldehyde. Results, expressed as percent stability, represent the ratio of 30% sucrose vs. 50% sucrose counts [the 50% sucrose counts being those shown in A]. ●, labeled MAP-microtubule seeds and unlabeled tails; ▲, unlabeled MAP-microtubule seeds and labeled tails.

We conclude that the rate of MAP redistribution is low at steady state, and coordinate with the rate of treadmilling. There is no rapid MAP redistribution, attributable to either MAP sliding or equilibrium exchange at steady state.

### Control of MAP Stabilization of Microtubules

We have demonstrated in several ways that MAP addition to and stabilization of microtubules is a rapid process. Addition of MAP-microtubule protein to assembled PC-tubulin yields maximal stabilization of the assembled polymers in highly diluted conditions, in 5 min (Fig. 4A). With protein concentrations compatible with assembly, MAP-microtubule protein addition onto MAP-microtubules causes an asymmetric distribution of MAPs on the polymer (Fig. 5A) indicating a rapid and irreversible MAP binding reaction on the pre-existing polymers. The rapidity of MAP binding and stabilization in such concentrated protein solutions is too rapid to be accurately measured by our current methods, but another experiment shows dramatically how rapid a process it can be. PC-tubulin microtubules are extremely labile to 25  $\mu$ M PLN, disassembling 50% in <1 min (Fig. 7). If, on the other hand, purified MAPs are pre-mixed with the PLN and the two added together, the MAPs (6% of the final protein mass) cause (Fig. 7) the instantaneous and nearly quantitative stabilization of these microtubules to drug-induced disassembly.

Although MAP-containing regions of microtubules are indefinitely stabilized under our assay conditions, the binding of MAPs does not produce a "frozen" state. These regions are slowly labile to dilution disassembly in 30% sucrose (Fig. 2) and to cold temperature in 50% sucrose (not shown). Further, as shown below, they respond by disassembling in the presence of calcium. We have also found (below) that PC-microtubules are not stabilized on mixture with MAPs that have previously been phosphorylated.

To determine the response to calcium, we assembled either PC-microtubules or MAP-microtubules with [ $^3$ H]GTP, then diluted them into 50% sucrose containing the free calcium concentrations indicated. After 30 min, the microtubules were filter assayed for stability by our standard procedure. PC-microtubules are labile to calcium beginning at sub-micro-

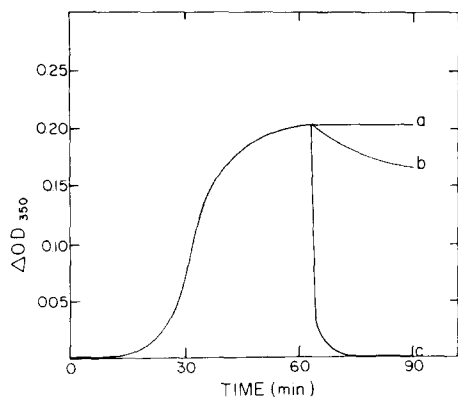


FIGURE 7 Assay of MAP stabilization of PC-tubulin microtubules. PC-tubulin was assembled (1.5 mg/ml; 30°C) in DG-MME buffer, and the assembly monitored by turbidity measurement at 350 nm. At steady state PLN (25  $\mu$ M) was added either with (b) or without (c) MAP protein. Final MAP concentration was 6% of tubulin. A represents a control to which only buffer has been added. All samples have been diluted to the same extent at steady state.

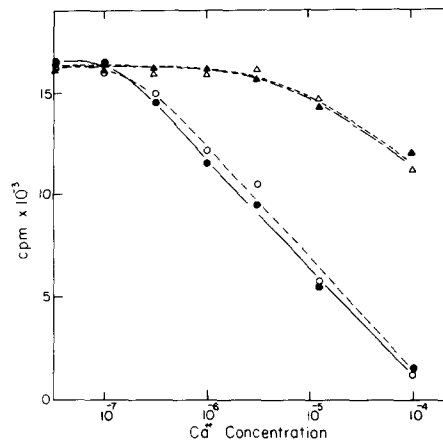


FIGURE 8 Response of microtubules to calcium and calmodulin. Either PC-tubulin or MAP-microtubule protein (6% MAPs) was assembled in DG-MME buffer under standard conditions at 1.5 mg/ml protein concentration. During assembly, [ $^3$ H]GTP was included to label the microtubules. At steady state they were diluted 25-fold in 50% sucrose-MME containing the indicated amounts of free calcium. Free calcium levels were calculated as reported previously (25). Calmodulin, when present, was in molar stoichiometry to tubulin. After 30 min of exposure to the experimental conditions, the microtubule samples were brought to 1% glutaraldehyde and processed for filter assay by the usual procedures. All results are reported as cpm (minus 600 cpm blanks). ●, PC-tubulin with calcium alone; ○, PC-tubulin with calcium and calmodulin; ▲, MAP-microtubules with calcium alone; Δ, MAP-microtubules with calcium and calmodulin.

molar concentrations (Fig. 8), but MAPs (6% level) protect microtubules until calcium concentrations in excess of 5  $\mu$ M are reached (Fig. 8).

These experiments were also run in the presence of calmodulin, to assess the role of this protein in MAP-dependent microtubule stability using this sensitive assay system. Calmodulin at a concentration equimolar to tubulin yielded no specific response with either PC-microtubules or MAP-containing microtubules (Fig. 8). Assuming calmodulin binds only to MAPs (19, 24, 25), it was ~20–40-fold in molar excess over MAP-2 or tau when added to MAP-microtubule protein. The calmodulin was independently assayed for its activity by measuring its destabilization of cold stable microtubules, with results equivalent to those previously published (26).

Phosphorylation of MAPs substantially diminishes their ability to protect microtubules against dilution disassembly. MAP-microtubule protein was incubated for various times with 0.5 mM ATP, with 0.5 mM ATP in the presence of the catalytic subunit of cAMP-dependent protein kinase II, or with 0.5 mM 5'-adenylylimidodiphosphate in the presence of protein kinase. At the indicated times, the reaction was quenched with 10 mM EDTA. This MAP-microtubule protein was then 25-fold diluted into 50% sucrose buffer and mixed with preassembled PC-microtubules (also 25-fold diluted into 50% sucrose buffer). After 20 min, these microtubules were assayed for stability by our standard procedure. The result (Fig. 9) is clear. Phosphorylation of MAPs quantitatively diminishes the ability of MAPs to stabilize microtubules to dilution disassembly.

### DISCUSSION

Several proteins and groups of proteins associate with microtubules in vivo and in vitro. Most of these proteins have only

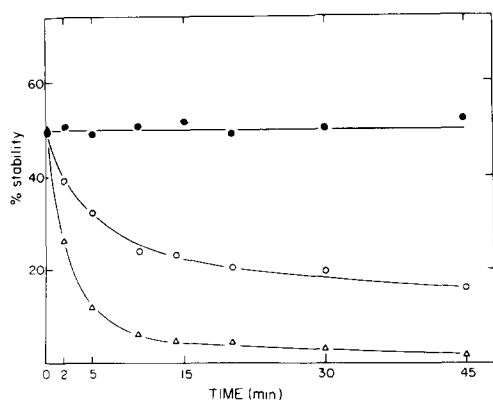


FIGURE 9 Effect of phosphorylation on MAP stabilization of microtubules. MAP-microtubule protein (25% MAPs) was incubated at 30°C for the indicated times in MME buffer supplemented with  $MgCl_2$  to 5 mM. The incubations contained either ATP or 5'-adenylylimidodiphosphate at 0.5 mM, and 1% (wt/wt of total protein) catalytic subunit of cAMP-dependent protein kinase, as indicated. At the indicated times, incubations were terminated by cooling to 0°C and adding 10 mM EDTA. PC-tubulin was then added, to yield a final MAP concentration of 6% and the MAP-microtubule protein was then diluted 25-fold into 50% sucrose-MME buffer. In parallel, PC-tubulin (1.5 mg/ml) was assembled in DG-MME buffer under standard conditions with  $[^3H]GTP$ , then diluted 25-fold in 50% sucrose-MME buffer. These labeled microtubules were then mixed in a 1:1 vol with the preincubated MAP-microtubule protein and incubated together. After 20 min, 1 ml of each sample was brought to 1% glutaraldehyde, while another 1 ml was diluted with MME to 30% sucrose (final concentration), incubated an additional 10 min, and finally cross-linked with 1% glutaraldehyde. Results are expressed as the percent of stability in 30% sucrose vs. 50% sucrose. ●, incubation with 5'-adenylylimidodiphosphate and catalytic subunit; ○, with ATP alone; △, with ATP and catalytic subunit.

vaguely understood physiological roles. Among these proteins are the MAPs that co-purify in constant stoichiometry with brain-derived microtubules through *in vitro* assembly cycles. These include two high molecular weight families, MAP-1, and MAP-2; and a group of four 55–68-kD proteins designated taus. MAP-1 is a three-subunit phosphoprotein of 350-kD, with one large subunit, and two of low molecular mass (27). MAP-2, another phosphoprotein (12), associates avidly with cAMP-dependent protein kinase (28) and is restricted in distribution to dendrites (29, 30). MAP-2 is reported to serve as linkage to neurofilaments (31), and may serve to cross-link and coordinate the two fibrous systems. This protein may also link microtubules to actin polymers (32, 33). The taus are present in low abundance on brain-derived microtubules (20). Each tau subunit is highly elongated and may in consequence associate with as many as four tubulin subunits within the polymer (34, 35). Each of these MAPs may act to stabilize the polymer, and so may enhance both the initiation and the final extent of assembly.

#### Generation of Microtubule Subclasses, and The "Dynamic Instability" Model

The findings we have presented here make clear some interesting properties of MAPs with respect to microtubule stabilization. MAPs bind with great rapidity to microtubules *in vitro*, and do not measurably exchange off of these polymers once bound. Neither do they, under the assay conditions

studied, exhibit any sliding behavior on microtubules. When MAPs are present in concentrations substantially below those required to saturate the polymer, the properties of MAPs described above have predictable consequences. MAPs will be unevenly distributed due to their rapid binding to the first formed polymer regions. At low concentrations they can thus effect a substantial stabilization of polymers in quite local domains. This substantial bias in distribution, combined with the failure of MAPs to readily exchange off of the polymers, can predictably produce the interesting effect, recently reported, of assembly in a distinct microtubule subpopulation during dilution-induced disassembly (14).

We can imagine this event occurring in the following way. Microtubules are assembled from a protein preparation containing a low concentration of MAPs. A portion of the MAP population induces assembly and the still-soluble MAP pool quickly coalesces on the first formed polymer regions. The MAP pool rapidly depletes and the remainder of assembly proceeds by PC-tubulin addition off of these "seeds." Since the MAPs do not redistribute, the initially stable regions remain indefinitely so.

Under such restrictive growth conditions, the microtubules would be quite long, unstable, and subject to shear. This would generate two distinct microtubule populations; one with no MAPs and highly unstable, and one with MAPs and relatively stable. On inducing disassembly by mild dilution, one would fall below the critical concentration for assembly of the unprotected polymers, leading to their precipitous decline. The rising titer of tubulin subunits would soon, however, exceed the postdilution critical concentration of the MAP-containing subpopulation, leading to its rapid assembly.

This explanation is, we believe, at least as reasonable as the explanation offered previously. Mitchison and Kirschner (14) have suggested that a GTP cap on microtubules could account for their data, but this explanation would require (a) the unique and stable distribution of GTP caps to only selective microtubule subpopulations, and (b) GTP to remain unhydrolyzed on its bound subunit for periods of time long in excess of the actual time required for the hydrolysis step (36).

Mitchison and Kirschner (14) report they have some MAP contamination in their preparation, as determined by antibody detection of MAPs in protein blots. We have reported here that even very small amounts of MAP protein can profoundly affect microtubule stability behavior, in a manner that is permanent with respect to a particular microtubule subpopulation. In preliminary studies we have found that ~1% MAP content is required to induce microtubule assembly to the extent reported by Mitchison and Kirschner, under their conditions.

If we are right, a specific prediction will be borne out. Paradoxical lengthening of microtubules upon dilution will not occur with strictly MAP-free microtubules, nor with MAP-saturated microtubules, but only in the special case where MAPs are in relatively low concentration. We are currently testing this possibility.

The effect of unequal distribution of MAPs on microtubules on the intracellular behavior of microtubules is another intriguing possibility. There may not only be different subpopulations of microtubules, but also differences of stability within a single microtubule. These differences, if stable, could lead to the establishment of different functional microtubule subpopulations.



We must add a caveat to the above analysis. Our experiments have been performed in assay buffers which contain 50% sucrose or 5% dimethyl sulfoxide and 10% glycerol. It is possible that these conditions can cause extraordinary stabilization of MAP binding to microtubules, and the indefinite stabilization of MAP-containing regions might not occur in the PIPES buffer used by Mitchison and Kirschner. Experiments currently in progress will, we believe, resolve this issue.

### *MAPs Compared with STOPs*

The characteristics found here for MAPs may be compared with those of STOPs. We have extensively investigated the behavior of STOP, which is apparently a 150-kD polypeptide tightly associated with, and creating, a subclass of microtubules that is uniquely cold stable (37). MAPs share with the STOPs the property of stabilizing microtubule regions to disassembly. STOPs differ from MAPs in being active at much lower relative concentrations and in being much more absolute a block. STOPs share with MAPs the property of binding rapidly to steady-state microtubules and in an effectively irreversible manner (23). As we have found with MAPs, STOP activity does not transfer from one microtubule to another in solution (23).

STOPs have a remarkable property, that of sliding on their host microtubule (23, 27). The ability to slide in an apparently diffusional manner is not apparently shared by the MAPs, at least under the assay conditions we have used here. We have described here an assay system which should enable us to determine if the conditions can be found that will enable MAP sliding on microtubules. Microtubules are an intracellular motility network upon which various organelles are transported (38). The sliding of one microtubule-bound protein suggests that others may slide as well, and may therefore create the microtubule-dependent motility that has been so extensively documented.

### *Phosphorylation Effect*

The stabilization of microtubules that we have observed here is not likely to be the simple product of a nonspecific electrostatic interaction between proteins. Phosphorylation of MAPs prior to their addition to microtubules totally abolishes their capacity to stabilize the polymers against dilution-induced disassembly. We have found, however, that the addition of exogenous protein kinase is necessary to obtain maximal inhibition of the MAPs.

Phosphorylation of MAP-2 and of tau have both been observed (12, 13, 39). MAP-2 phosphorylation causes a decrease in microtubule stability (40); a property recently reported for tau protein as well (41). In this assay system, with a mixture of MAP-2 and tau (both of which stabilize microtubules by our assay) (F. Pirollet, D. Job, and R. Margolis, unpublished results), it is evident that the phosphorylation effect on microtubule stability must be mediated through phosphorylation of both MAP-2 and tau.

It is clear that MAP phosphorylation leads to a destabilized interaction with microtubules. It is possible, therefore, that intermediate levels of phosphorylation lead to MAP equilibrium exchange on microtubules. We have not explored this possibility.

### *Calmodulin Effects on MAPs: Consequences for the Calcium "Flip-Flop" Mechanism*

We have used our assay system to determine if calcium and calmodulin would exhibit specific regulatory behavior with respect to MAP stabilization of microtubules. We have found that calcium causes a concentration-dependent destabilization of microtubules either in the presence or absence of MAPs, but that the presence of MAPs greatly diminishes the effect of calcium at any single concentration. The additional presence of calmodulin surprisingly causes no enhancement of calcium-dependent destabilization either in the presence or absence of MAPs. This result was obtained despite the fact that calmodulin was 20–40 times in molar excess to MAPs. The result contrasts with those we have obtained with similarly prepared microtubules, but in the additional presence of STOP. Calmodulin causes cold labilization of STOP-containing microtubules at calcium concentrations that alone have no effect on the stability of these microtubules (26). In previous assays where calmodulin was shown to destabilize cold labile microtubules *in vitro* (42), calmodulin was at least 100-fold in molar excess to MAPs, and probably was interacting in a low affinity, nonspecific manner with microtubules.

It has been shown that MAPs bind to  $\text{Ca}^{2+}$ -calmodulin (19, 24, 25). On the basis of this result, and the apparent calmodulin-dependent destabilization of microtubules, it was proposed that tau protein engaged in a "flip-flop" mechanism (43), either binding to microtubules or to calmodulin. However, it has not been demonstrated that  $\text{Ca}^{2+}$ -calmodulin actually caused any MAPs to dissociate from pre-assembled microtubules. Assuming MAP-dependent microtubule stability is not influenced by calmodulin and that MAPs bind calmodulin, one is left with two alternative choices. MAPs are capable of binding to calmodulin and to microtubules simultaneously, and may therefore serve as linkers between microtubules and membrane-associated calmodulin. Alternatively, calmodulin and tubulin compete for MAP binding, but calmodulin is only capable of sequestering MAPs if the MAPs are dissociated from microtubules by independent means, or are bound by calmodulin prior to their association with microtubules. We are currently testing to distinguish between these possibilities.

### *The Assay*

We have developed a sensitive assay to measure the distribution of MAPs on microtubules and on domains within a single microtubule. The assay relies on the ability of MAPs to stabilize microtubules against dilution-dependent disassembly when present in very low titers on the polymers. Microtubules may be labeled by [ $^3\text{H}$ ]GTP uptake into the polymer and the label may be introduced selectively in either MAP-containing or MAP-free regions. The assay is similar to that previously used to determine STOP protein distribution, equilibrium exchange, and sliding on polymers (23). It differs from this latter assay in its buffer conditions which have been chosen to maximize differences due to the particular stabilization properties of the MAPs. We have used glutaraldehyde successfully to trap [ $^3\text{H}$ ]GTP in microtubules, and to thus establish time points during assay. The fact that this protocol quantitatively measures the assembly state and stability of the polymer was established in Fig. 1. Our results may be com-

pared with those of Himes et al. (44), who reported substantial loss of [<sup>3</sup>H]GTP from microtubules on exposure to glutaraldehyde under certain conditions. We have found, in agreement with their results, that use of MME buffer, or of different glutaraldehyde concentrations, yielded a substantial loss of label. Proper selection of conditions, however, can produce a highly reliable glutaraldehyde-dependent assay.

This assay, in other variations, will be quite useful (a) in screening for other proteins with microtubule-stabilizing properties, (b) in further determining the physiological regulation of MAP stabilization of microtubules, and (c) in determining if conditions can be found in which MAPs can be induced to slide on the polymer.

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*Dedicated to Mathias Job (1969–1985), who took ill suddenly and tragically while this manuscript was in progress.*

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