

# Domains of Neuronal Microtubule-associated Proteins and Flexural Rigidity of Microtubules

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**Abstract.** Microtubules are flexible polymers whose mechanical properties are an important factor in the determination of cell architecture and function. It has been proposed that the two most prominent neuronal microtubule-associated proteins (MAPs), tau and MAP2, whose microtubule binding regions are largely homologous, make an important contribution to the formation and maintenance of neuronal processes, putatively by increasing the rigidity of microtubules. Using optical tweezers to manipulate single microtubules, we have measured their flexural rigidity in the presence of various constructs of tau and MAP2c. The results show a three- or fourfold increase of microtubule rigidity in the presence of wild-type tau or MAP2c, respectively. Unexpectedly, even low concentrations of MAPs promote a substantial increase in microtubule rigidity. Thus at ~20% saturation with full-length tau, a micro-

tubule exhibits >80% of the rigidity observed at near saturating concentrations. Several different constructs of tau or MAP2 were used to determine the relative contribution of certain subdomains in the microtubule-binding region. All constructs tested increase microtubule rigidity, albeit to different extents. Thus, the repeat domains alone increase microtubule rigidity only marginally, whereas the domains flanking the repeats make a significant contribution. Overall, there is an excellent correlation between the strength of binding of a MAP construct to microtubules (as represented by its dissociation constant  $K_d$ ) and the increase in microtubule rigidity. These findings demonstrate that neuronal MAPs as well as constructs derived from them increase microtubule rigidity, and that the changes in rigidity observed with different constructs correlate well with other biochemical and physiological parameters.

**M**ICROTUBULES are important components of the eukaryotic cytoskeleton that are involved in basic cellular activities such as cell shape determination, cell movement, mitosis, and organelle transport (for comprehensive reviews see Dustin, 1984; Hyams and Lloyd, 1993). Two major classes of proteins interact with microtubule polymers: molecular motors and microtubule-associated proteins (MAPs).<sup>1</sup> In contrast with motor molecules, MAPs bind to microtubules in a nucleotide-independent fashion. They copolymerize with microtubules through cycles of assembly and disassembly, stimulate their nucleation, and stabilize the polymer (Olmsted, 1991; Schoen-

feld and Obar, 1994; Mandelkow and Mandelkow, 1995). Two of the best-known families of MAPs are the tau proteins and MAP2. Both are expressed in neuronal cells, with the high molecular weight form of MAP2 being confined to dendrites and tau being confined to axons (Bernhardt and Matus, 1984; Binder et al., 1985; Tucker et al., 1988). They are derived from different genes (Lee et al., 1988; Lewis et al., 1988) and, in each case, alternative splicing gives rise to several isoforms (Himmler et al., 1989; Goedert et al., 1989; Kindler et al., 1990), the size of which may vary considerably. Despite these size differences, MAP2 and tau are similar in overall domain composition, including a set of three or four repeats of ~30 amino acids in length. The repeats and the flanking proline-rich and basic domain in the COOH-terminal half comprise the "assembly domain" that is responsible for binding to microtubules (Butner and Kirschner 1991; Brandt and Lee, 1993; Coffey et al., 1994; Gustke et al., 1994; Ludin et al., 1996). The role of the NH<sub>2</sub>-terminal half that does not bind to microtubules and that extends into the surround-

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1. *Abbreviations used in this paper:* MAP, microtubule-associated protein; EI, flexural rigidity.

ing medium ("projection domain") is less clear. Its size determines the separation between microtubules in a parallel array (e.g., Black, 1987; Chen et al., 1992; Weisshaar et al., 1992). In addition, it may serve as an anchoring device for cellular proteins, notably kinases and phosphatases (e.g., Mandelkow et al., 1992; Sontag et al., 1995). Whereas MAP2 and tau are almost exclusively expressed in neurons, other cell types contain the more ubiquitous MAP4 that possesses many of the same features, including the repeated sequences (West et al., 1991; Chapin and Bulinski, 1991).

Experiments in which MAP2 and tau have been expressed by transfection in nonneuronal cells have shown that both MAPs significantly influence microtubule stability and arrangement. In their presence microtubules are stabilized, form bundles, and become capable of supporting process formation in cells from which they are normally absent (Lewis et al., 1989; Takemura et al., 1992; Knops et al., 1991; Weisshaar et al., 1992; Edson et al., 1993). These studies suggest that tau and MAP2 play a crucial role in microtubule assembly and stability, and thus contribute to the integrity and dynamics of the cells in which they are expressed.

To serve a stabilizing and structuring function in the cell, a microtubule must be able to resist tensile and compressive forces. This capacity is reflected in its flexural rigidity, an important physical characteristic of these extended polymers. The flexural rigidity of single microtubules has recently been determined using a variety of approaches, including the analysis of thermal fluctuations in shape (Gittes et al., 1993; Venier et al., 1994; Mickey and Howard, 1995), measurement of the deformation induced by hydrodynamic flow (Dye et al., 1993; Venier et al., 1994; Kurz and Williams, 1995), and laser trap-induced flexing with (Kurachi et al., 1995) or without (Felgner et al., 1996) attached beads. These studies agree that microtubules are ~100 times more rigid than actin filaments, another major cytoskeletal filament of eukaryotic cells. Since MAPs are important elements in determining the biochemical, and perhaps also the physical, properties of microtubules in the cell, some of these studies also addressed the question of how MAPs affect microtubule rigidity. However, although the microtubule-supported processes that MAP2 induces in fibroblastic cells are demonstrably stiff (Ludin et al., 1996), the question whether individual microtubules are made more rigid by MAPs is still open.

In a previous study addressing the mechanical properties of microtubules, we have used optical tweezers to deflect a single microtubule attached to an axoneme (Felgner et al., 1996). From the quantitative analysis of the relaxation movement, the microtubule's flexural rigidity is determined. It is a straightforward (though by no means trivial) method to measure the stiffness of a biopolymer. In the present study we have determined the influence of MAP2 and tau on the flexural rigidity of single microtubules in vitro. Unphosphorylated recombinant proteins purified from bacterial extracts were used to avoid complications introduced by variations in phosphorylation that affect MAP-microtubule interactions (Brugg and Matus, 1991; Verde et al., 1991; Drechsel et al., 1992; Trinczek et al., 1995). To determine the contribution of different microtubule-binding domains of tau and MAP2 to microtu-

bule rigidity, we have tested a number of constructs that varied in the composition of subdomains in the microtubule-binding (assembly) domain. The results show that all constructs tested increase microtubule rigidity and that the contribution of certain subdomains correlates well with their strength of binding to microtubules.

## Materials and Methods

### Optical Tweezers Setup and Image Processing

The setup used for the construction of the laser trap is described in detail in Felgner et al. (1995, 1996). Briefly, the expanded beam of a continuous wave Nd:YAG laser (Spectron, Rugby, UK) is coupled into an upright microscope using a 100×/1.3 NA objective (Zeiss, Oberkochen, Germany). A laser output power of 1.5 W is coupled into the epifluorescence illumination path of the microscope, 40% of which reaches the sample. Whereas the laser beam is fixed relative to the optical system, the specimen chamber is translated relative to the beam using a motorized microscope stage (Maerzhaeuser, Wetzlar, Germany). A Newvicon camera (Hamamatsu, Herrsching, Germany) feeds the microscope image into a Macintosh IIfx computer (Apple Computer Inc., Cupertino, CA). For image processing details, see Felgner et al. (1996).

### Preparation of Microtubules and Axonemes

Microtubules were prepared from phosphocellulose-purified pig brain tubulin according to the procedure of Shelanski et al. (1973), with modifications, including MAP-depleting steps, according to Mandelkow et al. (1985). Axonemes of *Chlamydomonas reinhardtii* were prepared according to Witman (1986) and depleted of dynein and other endogenous proteins by salt extraction (King et al., 1986). Polylysine coverslips were prepared by applying a drop of 1 mg/ml poly-L-lysine in water to a coverslip for 30 s, followed by rinsing with water and air drying. Axonemes were attached to polylysine-coated coverslips, followed by the addition of tubulin (1 mg/ml) plus GTP (1 mM) in a buffer consisting of 100 mM Pipes, 2 mM MgCl<sub>2</sub>, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 10 μg/ml trypsin inhibitor, 10 μg/ml TAME, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 10 μg/ml aprotinin, pH 6.9. The preparations were incubated at 30°C for 10 min, which yielded axoneme-nucleated microtubules of 5–20 μm in length. MAPs or tau constructs were added at the desired concentration (see below), and the coverslip was placed on a slide and sealed with VALAP (vaseline/lanoline/paraffin = 1:1:1). The temperature in the chamber on the microscope stage during observation was 24–27°C.

### Adsorption to the Glass Surface and Levels of Saturation of the Microtubules by MAPs

To determine eventual losses of tubulin and MAPs in our experimental setup, we have measured their adsorption to the polylysine-covered glass surface. 10 μg protein in 100 μl buffer was applied to an 18 × 18-mm coverslip as a thin film and incubated for 30–60 min in a wet chamber. The solution was removed and the protein concentration was measured, taking into account that not all of the solution could be recovered from the coverslip. We find that 1.3 ± 0.4 μg of tubulin (*n* = 9) is adsorbed to the surface. This corresponds to one molecule per 42 nm<sup>2</sup>, a value that is in reasonable agreement with the surface area covered by one tubulin molecule (16–32 nm<sup>2</sup>), assuming dimensions of tubulin of 4 × 4 × 8 nm. The adsorption of MAPs is 0.6 ± 0.2 μg (*n* = 12) for a construct of average size (40 kD). If a coverslip is incubated with tubulin first, followed by the addition of MAPs, the adsorption of MAPs is reduced to <0.1 μg. This is important because in our experiments a coverslip is incubated with tubulin first (to allow for polymerization onto the axonemes) before the addition of MAPs in a 2-μl vol. The coverslip is then placed on a slide. On the slide surface, we assume tubulin and MAPs adsorb with equal efficiency. The total losses therefore are 1.3 μg (coverslip) plus half of 1.3 μg (slide) for tubulin, and <0.1 μg (coverslip) plus half of 0.6 μg (slide) for MAPs. These losses of 2 μg total for tubulin and 0.4 μg total for MAPs were taken into account in calculating the true molar ratio of MAPs to tubulin and hence the level of saturation of microtubules by MAPs.

The molar ratios of MAPs to tubulin applied to the coverslip were 1:1 for full-length tau and MAP2c constructs, 30:1 for the low affinity tau constructs K10 and K18, 0.4:1 for the high affinity construct T8R2, and 1.5:1

Pure	K18	K23	K10	K11	K30	K16	H23	K29	H40	2c3	2c	MM	2d	T8R2	
1%	1%	1%	1%	1%	1%	1%	1%	1%	1%	1%	1%	1%	1%	1%	Taxol
1%	1%	1%	1%	1%	1%	1%	1%	1%	1%	1%	1%	1%	1%	1%	Pure
		no	no	1%	1%	1%	1%	1%	1%	1%	1%	1%	1%	1%	K18
		no	5%	5%	5%	5%	5%	1%	1%	1%	1%	1%	1%	1%	K23
			5%	5%	5%	5%	5%	1%	1%	1%	1%	1%	1%	1%	K10
			no	no	no	no	no	1%	1%	1%	1%	1%	1%	1%	K11
			no	no	no	no	no	1%	1%	1%	1%	1%	1%	1%	K30
							no	no	1%	1%	1%	1%	1%	1%	K16
							no	no	1%	1%	1%	1%	1%	1%	H23
								no	5%	1%	1%	1%	1%	1%	K29
									1%	1%	1%	1%	1%	1%	H40
										no	no	1%	1%	1%	2c3
											no	no	1%	1%	2c
												no	1%	1%	MM
													1%	1%	2d

**Figure 1.** Statistical analysis using the nonparametric Wilcoxon test. *H23* and *H40*, constructs HT23 and HT40, respectively; *MM*, mixture of brain MAPs; *2c*, *2d*, and *2c3*, MAP2 isoforms; *Pure* and *Taxol*, microtubules prepared from phosphocellulose-purified tubulin and taxol-stabilized microtubules, respectively; 1%, 5%, and no, levels of significance.

for the others. The losses due to adsorption described above were subtracted subsequently. This leads to levels of saturation of microtubules by the various tau constructs of between 79 and 93% (calculated according to Gustke et al., 1994); an exception is K23, where only 65% saturation is reached for technical reasons. The errors due to pipetting are 1–3%. Nearly complete saturation of microtubules by MAPs was not attempted because it would require unreasonably high concentrations of several of the lower affinity constructs. In cells, the level of saturation by tau is estimated to be 40–70% (Drubin et al., 1985; Drubin and Kirschner, 1986).

### Cloning and Expression of Tau Constructs

Recombinant full-length tau and other constructs were cloned in the pNG2 expression vector and expressed in *Escherichia coli* as described by Biernat et al. (1992) and Gustke et al. (1994). The clones were derived from isoforms of human tau (Goedert et al., 1989). The numbering of residues follows that of human tau HT40, the largest of the six isoforms in the central nervous system (441 residues). Variations included the following: changes in the number of repeats, changes in the targeting domains flanking the repeats, omission of the projection domain, excision of the repeats, and others (see Fig. 4).

### Preparation of MAP2 and Constructs

MAP2c and deletion mutants were prepared as described by Ludin et al. (1996). Briefly, appropriate cDNAs were subcloned into the bacterial expression plasmid pET3d and grown in *E. coli* BL21(DE3). Protein expression was induced by the addition of 1 mM isopropylthio- $\beta$ -D-galactoside for 3 h, and the protein was extracted and purified as described in Ludin et al. (1996).

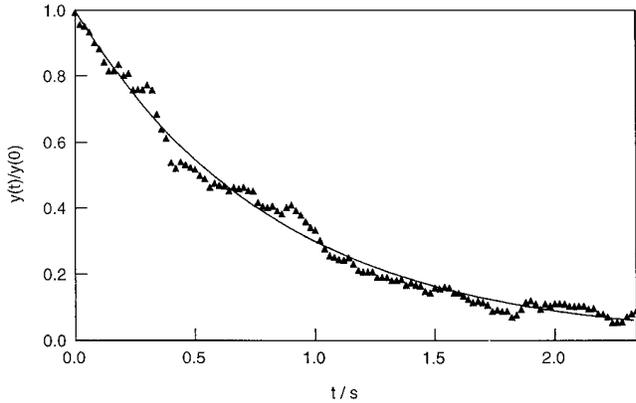
### Statistical Analysis

A nonparametric Wilcoxon test was carried out according to Bronstein (1985) to compare the flexural rigidities of microtubules in the presence of different constructs of tau. In most cases, the hypothesis that two populations of microtubules have the same flexural rigidity could be rejected with a significance level of 1 or 5% (Fig. 1). No significant differences were found for the MAP2 constructs.

## Results

### Measurement of Flexural Rigidity

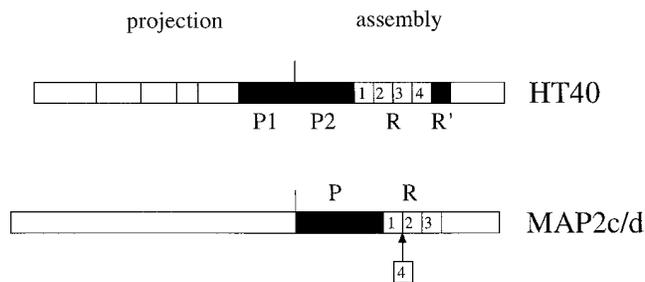
The procedure used here is based on the calculation of frictional hydrodynamic forces acting on a single moving



**Figure 2.** Representative experiment showing the time course of the relaxation movement of a microtubule prepared from pure tubulin (length 13  $\mu\text{m}$ ) relaxing from its deflected position ( $y(0) \sim 4 \mu\text{m}$ ) to its resting position. The triangles show measured values with a time resolution of 50 Hz, and the line represents a fit to the expected exponential decay from which EI is calculated.

microtubule. A microtubule anchored at its minus end at an axoneme is captured directly near its free end with the laser tweezers and deflected in a direction perpendicular to its long axis and parallel to the coverslip surface. The laser power is then switched off, allowing the microtubule to relax back to its straight resting position. The flexural rigidity can be calculated from the relaxation movement, which is determined by the elastic force of the microtubule that drives it back and the hydrodynamic force of the medium that resists the moving microtubule. An example is shown in Fig. 2. The details of the theoretical considerations on which the analysis of this movement is based are outlined in Felgner et al. (1996). For a microtubule without MAPs, the flexural rigidity (EI; E = Young's modulus, I = geometrical moment of inertia) is  $\sim 4 \times 10^{-24} \text{ Nm}^2$  (see Fig. 4). When microtubules are stabilized by taxol, we find the value of EI to decrease by a factor of 2–3, suggesting that taxol, which makes microtubules less dynamic, also makes them less rigid (Felgner et al., 1996).

Various structural, biochemical, and physical factors that affect the determination of the flexural rigidity in our approach have already been discussed (Felgner et al., 1996) and need not be reiterated here. Some of these factors introduce uncertainties that affect the determination of EI, leading to either an underestimation or overestimation of its true value. These uncertainties have to be taken into account when comparing our results with those obtained using other methods. However, even if this is done, some discrepancies remain. For example, the rigidity of untreated microtubules determined from thermal fluctuations by Mickey and Howard (1995), or from flow-induced deformation by Kurz and Williams (1995), is about seven to nine times higher than ours. On the other hand, Venier et al. (1994) and Tran et al. (Tran, P.T., S.F. Parsons, R. Sterba, Z. Wang, M.P. Sheetz, and E.D. Salmon. 1995. *Mol. Biol. Cell.* 6:260a) found a value of EI very similar to ours. These discrepancies are difficult to explain at present, although it cannot be excluded that they arise from intrinsic differences in the microtubules used or certain features of the assay conditions. Regardless, the comparison between



**Figure 3.** Simplified diagrammatic representation of the domain organization of tau (the largest isoform *HT40*) and MAP2c. The 31–amino acid repeats are numbered. In MAP2d a fourth repeat is inserted between repeats 1 and 2. *P1*, *P2*, and *P*, proline-rich domains. *R'*, pseudorepeat.

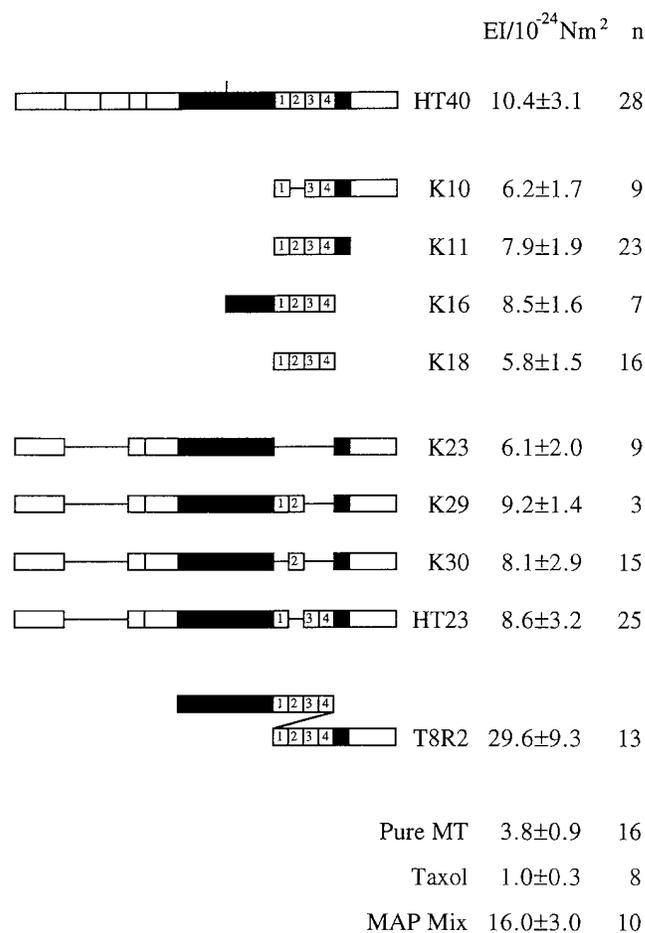
MAP-containing and pure microtubules undertaken in this study is not affected by these discrepancies because identical procedures were used in all experiments.

### Microtubule Rigidity in the Presence of Different Tau Constructs

The COOH-terminal assembly domain of tau can be divided into a number of subdomains with different characteristics (Fig. 3). The most notable feature is the presence of four repeats 31 or 32 amino acids in length (Lee et al., 1988; Himmler et al., 1989; Goedert et al., 1989). A fifth, less conserved repeat-like domain (*R'*) is followed by a COOH-terminal domain that apparently lacks distinctive features. A proline-rich domain  $\text{NH}_2$ -terminal to the repeats can be subdivided into portions located on either the projection (*P1*) or the assembly domain (*P2*). The domains flanking the repeats can be considered targeting domains that fix tau on the microtubule surface, while the repeats enhance microtubule assembly (“jaws model”; Gustke et al., 1994).

To determine the relative contributions of tau domains to the increase in microtubule stiffness, we tested a number of tau constructs. The data are summarized in Fig. 4. As outlined in Materials and Methods, the molar ratio of a given MAP construct to tubulin was adjusted so that the level of saturation of microtubules by MAPs was ~80–90% (except K23, where 65% saturation was reached). This is higher than the presumptive level of saturation of tau in vivo, which can be estimated to be 40–70% (Drubin and Kirschner, 1986). However, we reasoned that using a higher level of saturation may help identify potentially weak effects of these constructs on microtubule rigidity.

The flexural rigidity of microtubules with bound full-length tau is almost three times higher than that of pure microtubules. All tau constructs lacking one or more subdomains in the assembly portion caused microtubule stiffness to decrease relative to full-length tau. Apparently, all subdomains of the assembly domain contribute to microtubule rigidity, albeit to different extents. Thus the four repeats alone (K18) increased microtubule rigidity only slightly, but, when combined with either the *P2* region (K16) or the pseudorepeat (K11), microtubule rigidity more than doubled. Constructs in which the repeat domains were deleted but the flanking domains were main-



**Figure 4.** Tau constructs and microtubule rigidity. For domain organization see Fig. 3. The figure shows the flexural rigidity (*EI*) of the different constructs and the number of experiments (*n*). The values for MAP-free microtubules (*Pure*), taxol-stabilized microtubules (*Taxol*), and microtubules in the presence of a mixture of brain MAPs (*MAP Mix*) are also included for comparison.

tained yielded complementary results. The flanking domains alone already contributed substantially to microtubule rigidity (K23); in combination with one or more repeat domains, it increased even more (K29, K30, and HT23). As a rule, the differences in microtubule rigidity observed in our measurements were statistically significant (Fig. 1) for all pairs of constructs that differed by more than 2 U of *EI* ( $10^{-24} \text{Nm}^2$ ).

An unusual observation was made with a construct in which the four repeat domains are doubled up (T8R2). It increased microtubule stiffness far beyond a value that might be expected on the basis of the observations made with the deletion constructs. Microtubule rigidity was almost three times higher than that with full-length tau and almost eight times that of pure microtubules. Visual inspection of these microtubules clearly demonstrated that their fluctuations in shape as a result of thermal motion were greatly suppressed.

Several other constructs that we also wished to include in this analysis unfortunately led to substantial microtubule bundling and therefore could not be tested.

**Table I. Flexural Rigidity at Different Levels of Saturation by Full-Length Tau (HT40)**

Calculated level of saturation	EI	<i>n</i>
%	$10^{-24}/\text{Nm}^2$	
0 (pure MTs)	$3.8 \pm 0.9$	16
2	$4.5 \pm 1.5$	14
18	$8.9 \pm 1.3$	8
48	$9.4 \pm 2.6$	14
85	$10.4 \pm 3.1$	28

EI, flexural rigidity  $\pm$  SD; *n*, number of experiments.

### Levels of Saturation of Microtubules by Tau

To determine how the level of saturation of a microtubule with a given tau construct affects its flexural rigidity, experiments were made with full-length tau (HT40) at concentrations leading to ~2, 18, and 48% saturation, in addition to the experiments at 85% saturation listed in Fig. 4. As shown in Table I, even a relatively low level of saturation (18%) already led to a substantial increase in microtubule rigidity. At 48 or 85% saturation, the additional increase in rigidity is comparatively small. These observations were supported by findings with other constructs. For example, T8R2 was also used at a concentration that leads to 99% saturation of microtubules, but no difference was found in microtubule rigidity (average  $29.2 \pm 4.8$  in units of  $10^{-24} \text{ Nm}^2$ ;  $n = 25$ ) compared with experiments at 84% saturation (see Fig. 4). Similarly, construct K10, at a concentration that saturated microtubules to only 40%, increased microtubule rigidity to a value ( $6.0 \pm 1.3$ ;  $n = 17$ ) almost identical to that measured at 93% saturation (Fig. 4). K18 seemed to be somewhat less effective as 34% saturation caused microtubule rigidity to increase to  $4.9 \pm 1.1$  ( $n = 14$ ). These findings suggest a nonlinear relationship between microtubule saturation by MAPs and flexural rigidity, where partial saturation may already lead to nearly maximal microtubule stiffening.

### Microtubule Rigidity in the Presence of MAP2

MAP2c is a low molecular weight isoform of MAP2 that is expressed primarily in developing brain (Garner and Matus, 1988). Its COOH-terminal microtubule-binding domain begins at around amino acid 230 and encompasses a proline-rich subdomain and three imperfect tandem repeats of 31 amino acids (Fig. 3) that are homologous to the repeats found in tau. The entire assembly domain of MAP2c is almost exactly as long as that of tau. Nevertheless, our measurements of the rigidity of microtubules in the presence of MAP2c demonstrate an increase of ~50% above that of tau (Table II). In comparison with MAP2c, the recently discovered splice variant MAP2d, which contains a fourth conserved repeat inserted between the first and second (Doll et al., 1993; Ferhat et al., 1994), did not significantly increase microtubule rigidity. Likewise, microtubules complexed with the deletion construct MAP2c3, which contains only one of the 31-amino acid repeats from the COOH-terminal binding domain (Ludin et al., 1996), fell within the same range. All three constructs resulted in microtubules whose flexural rigidity was not significantly different from that of microtubules in the presence of a

**Table II. Flexural Rigidity in the Presence of MAP2 Isoforms**

Isoform	EI	<i>n</i>
	$10^{-24}/\text{Nm}^2$	
MAP2c	$15.1 \pm 3.3$	18
MAP2d	$16.1 \pm 2.7$	8
MAP2c3	$14.5 \pm 3.8$	16
Pure MT	$3.8 \pm 0.9$	16
HT40	$10.4 \pm 3.1$	28
MAP mix	$16.0 \pm 3.0$	10

MAP2d has an additional repeat inserted between repeats 1 and 2; MAP2c3 has repeat 3 only. The values for pure tubulin microtubules, and microtubules in the presence of HT40 or mixture of brain MAPs, are also included for comparison.

EI, flexural rigidity  $\pm$  SD. *n*, number of experiments.

mixture of brain MAPs, which presumably include various isoforms of MAP2, tau, and other MAPs. This finding suggests that the effect of various MAPs on microtubule stiffness are not additive and dominated by the high molecular weight MAPs.

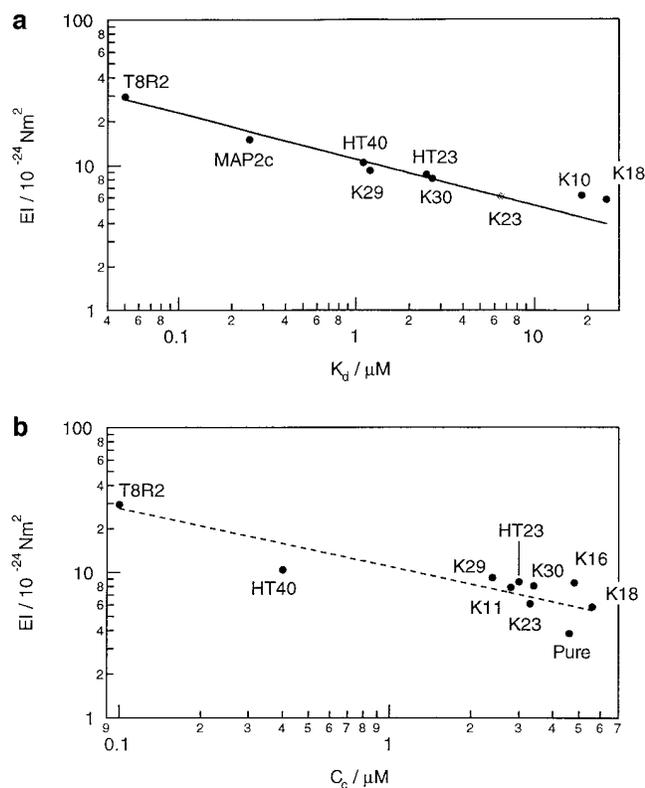
### Relationship with Other Parameters of MAP–Microtubule Binding

We compared the ability of tau constructs and MAP2c to alter microtubule rigidity with their binding to microtubules (dissociation constant,  $K_d$ ) and their influence on microtubule dynamics (critical concentration at the plus end,  $C_c$ ) determined earlier (Gustke et al., 1994; Trinczek et al., 1995; Illenberger et al., 1996). An excellent correlation was found with the binding affinity for microtubules (Fig. 5 a), a high value of EI corresponding to a low dissociation constant. A less perfect correlation also existed with the critical concentration for tubulin assembly at the plus end (Fig. 5 b), which is lower for constructs that induced a higher rigidity. Only weak correlations or none at all were found with other parameters of microtubule dynamics such as the frequencies of catastrophe and rescue, the nucleation rate, or the initial rate of assembly in solution (not shown).

### Discussion

Using optical tweezers as a micromanipulator to bend microtubules, we determined their flexural rigidity from the return movement to the resting position. We find that two types of MAPs, tau and MAP2c, both increase microtubule rigidity. Moreover, deletions of subdomains in the microtubule-binding region decrease microtubule rigidity in the case of tau but appear to have little effect in the case of MAP2c.

The subdomains of the microtubule-binding segment of tau all contribute to the buildup of microtubule rigidity, although the potency of their contribution varies. Thus, unexpectedly, the four repeat domains, which are believed to be important for microtubule binding (Lee et al., 1988; Himmler et al., 1989), and which make up half of the assembly domain, increase microtubule rigidity only marginally when used alone. However, in combination with either one of the flanking subdomains, rigidity is nearly as high as with full-length tau, supporting the jaws model for tau binding to microtubules (Gustke et al., 1994). These findings are also consistent with studies on MAP2c trans-



**Figure 5.** (a) Correlation between microtubule rigidity ( $EI$ ) and microtubule binding ( $K_d$ ) of various tau constructs and MAP2c. The plot demonstrates a good correlation between MAP binding and microtubule stiffness. The value for K23 is shown in gray because with this construct only 65% saturation of microtubules is reached, whereas all other constructs were used at levels of saturation  $>79\%$ . However, this may not make that much of a difference as even lower levels of saturation may cause close to maximal increases in microtubule rigidity (see Table I). (b) Relationship of microtubule rigidity ( $EI$ ) to the critical concentration of tubulin assembly at the plus end ( $C_c$ ) in the presence of different MAP constructs. In both *a* and *b*, lines were fitted by linear regression.

ected into cultured cells that show that the repeat domain itself binds poorly but, in combination with flanking sequences, binding as well as bundling and process formation are greatly improved (Ferralli et al., 1994). Conversely, the potential of the flanking regions of tau alone (K23) to increase microtubule rigidity is moderate, but it is enhanced in a stepwise fashion by the addition of one or more repeat domains, suggesting that each of the repeat domains makes a significant contribution. In MAP2c, on the other hand, the addition or deletion of repeat domains has no significant effect on microtubule stiffness, although it is conceivable that a potentially small contribution could not be detected with the methods used in this study.

How tau and MAP2c interact with microtubules is not known mainly because the molecular structure of these molecules can not yet be determined. In solution, tau has very little secondary structure and behaves as if it were a random polymer (Schweers et al., 1994). Consequently, it is unclear how much tau and MAP2c increase the hydrodynamically effective diameter of microtubules because

the shape and extension of the projection domain are unknown. It was not possible to test the influence of the projection domain on  $EI$  directly using constructs with identical assembly domains but with or without the projection domain. However, even if the MAP projections were to double the apparent diameter of the microtubules, which probably is an overestimation, flexural rigidity would be underestimated by 20% at most, as calculated by Felgner et al. (1996).

It is unlikely that some of the differences in microtubule rigidity observed here are caused by uneven distribution of MAPs along microtubules due to, e.g., cooperative binding. An overwhelming body of evidence collected in several laboratories suggests that MAPs are evenly distributed along a microtubule. Even though they may bind tightly, MAPs can still redistribute quickly, owing to fast on and off rates. MAP decoration of microtubules appears to be homogeneous even at low MAP/tubulin ratios (Murphy and Borisy, 1975; Sloboda and Rosenbaum, 1979; Stearns and Brown, 1979; Olmsted et al., 1989; Kowalski and Williams, 1993). Evidence for cooperative binding of MAP2 to microtubules *in vitro* is, at best, controversial (Wallis et al., 1993; Coffey and Purich, 1995), and with tau it is rather unlikely (Biernat et al., 1993; Gustke et al., 1994). Thus, we are reasonably confident that in the experiments reported here MAPs are distributed homogeneously along the length of a microtubule at all concentrations used.

All subregions of the assembly domain appear to contribute to microtubule binding, though to different extents. Binding is more efficient if the repeat domains plus one or both of the flanking regions are involved (Gustke et al., 1994). Remarkably, the same applies to the effect on microtubule rigidity. A good correlation with microtubule binding is revealed (Fig. 5 *a*), strongly supporting the conclusion that efficient binding also promotes efficient microtubule stiffening, with certain combinations of subdomains being more favorable than others.

Thus, the repeats alone (K18), which bind weakly to microtubules also increase microtubule stiffness only marginally, suggesting that they cannot be considered a "microtubule-binding domain" by themselves. Conversely, full-length tau or the series of constructs with both flanking domains and varying numbers of repeats (HT23, K23, K29, and K30) binds strongly and also increases microtubule rigidity significantly. As tight binding of a tau construct usually implies a high potential for promoting microtubule growth (Trinczek et al., 1995), the correlation between the potential to increase rigidity and the ability to lower the critical concentration for assembly (Fig. 5 *b*) is not surprising.

It may be premature to interpret the observed relationships in terms of molecular models for tau or MAP2c binding. The minimal conclusion is that the interaction of these MAPs with microtubules leads to an increase in microtubule rigidity; how well a construct also promotes microtubule assembly may enter as a supplementary consideration. In agreement with this conclusion, only weak correlations, or none at all, are found with parameters of microtubule dynamics such as the rates of catastrophe and rescue (Gustke et al., 1994; Trinczek et al., 1995). Thus, it seems that the influences of different MAPs on param-

ters of microtubule assembly and the mechanical properties of the polymer, respectively, are largely independent.

Interestingly, although the length and subdomain organization of the microtubule-binding segment of both tau and MAP2c are remarkably similar, MAP2c is significantly more potent in promoting microtubule rigidity. This may be related to the potency of the third repeat in MAP2. In cell transfection assays, this single 31-amino acid domain is sufficient to confer microtubule binding on the rest of the MAP2 sequence as well as the ability to support process outgrowth (Ludin et al., 1996). Processes formed with single-repeat MAP2c3 show comparable stiffness to those formed by cells expressing full-length MAP2c (Ludin et al., 1996). This is confirmed by the physical measurements made on single microtubules in this study. Together these data indicate that the successive binding of MAP repeats to neighboring tubulin subunits in the wall of the microtubule lattice is neither a necessary nor major feature of microtubule stiffening by MAPs. Further experiments using different MAP2c constructs are required to probe the mechanism by which a single MAP repeat, perhaps in association with other MAP2 sequence elements, acts to induce stiffening.

Our findings on the influence of MAPs on microtubule flexural rigidity are in partial agreement with studies from other laboratories. The experiments of Kurachi et al. (1995), though clouded by an unexpected dependence on microtubule length, and those of Tran et al. (Tran, P.T., S.F. Parsons, R. Sterba, Z. Wang, M.P. Sheetz, and E.D. Salmon. 1995. *Mol. Biol. Cell.* 6:260a.) both demonstrate, as we do, a large difference in the rigidity of microtubules in the presence or absence of MAPs. On the other hand, Mickey and Howard (1995) and Kurz and Williams (1995) conclude that the rigidity of single microtubules is only marginally increased by the binding of tau or a mixture of MAPs, respectively. While there is at present no satisfying explanation for these discrepancies, it is evident from the study reported here that microtubule rigidity does not change at random with different tau constructs but in a biologically consistent and interpretable manner. Moreover, the changes we observe with different tau constructs and MAP2c correlate well with other physiological parameters such as their strength of binding to microtubules. These factors lend strong support to the conclusion that MAPs do indeed increase microtubule rigidity.

How do MAPs and MAP constructs modulate microtubule rigidity? Two mechanisms (that are not mutually exclusive) can be envisioned. In the first, the assembly domains of tau or MAP2 are proposed to associate with the microtubule exterior in such a way that two or more tubulin subunits are interlinked. This would limit the freedom of interdimer motions during the bending of a microtubule and thus increase microtubule stiffness (Matus, 1994). Alternatively, the binding of MAPs (or fragments thereof) induces a conformational change in the tubulin dimer that either affects interdimer binding or puts the dimer in a strained conformation, analogous to that produced by GTP hydrolysis (Caplow et al., 1994; Mickey and Howard, 1995); both these processes may help stiffen the polymer (Bereiter-Hahn, 1978; Mandelkow et al., 1991). The magnitude of the conformational change promoted by the binding of a given MAP construct to microtubules and,

hence, the resulting effect on microtubule stiffness would depend on the length and domain organization of the construct. At present it is not possible to distinguish between these interpretations. As there is no strong evidence for cooperative binding of tau to microtubules, the pronounced effect of a low concentration of tau (18% saturation causes 85% of the maximal stiffening effect; see Table I) is unexpected and intriguing. It seems to suggest that the actions of individual MAP molecules on microtubules are not merely additive, with each molecule contributing an equal share until full saturation promotes maximal stiffening. Rather, subsaturation binding may induce conformational changes that affect more than just the tubulin subunits to which a MAP molecule binds. How this is achieved remains to be determined.

What are the cell biological consequences of MAP-induced microtubule stiffening? In neurons, the length of cell processes (axons and dendrites) may exceed the diameter of the cell body many hundredfold. This extreme cell asymmetry requires mechanisms that confer mechanical stability to these processes. Microtubules may well contribute to the morphological stability of these cell processes, and MAPs, in turn, may well contribute to the mechanical stability of microtubules. There are three properties that MAPs could confer on microtubules to enhance their ability to act as elements of structural support: reduction of microtubule dynamic instability, increase in microtubule length, and increase in microtubule rigidity. Of these, stabilization by reducing the growth and shrinkage of microtubules is less likely as MAP2 and tau do not significantly affect microtubule dynamic instability (Trinczek et al., 1995; Kaech et al., 1996). Increased microtubule length and rigidity, on the other hand, are important factors, as demonstrated by cell process formation in MAP-transfected nonneuronal cells (e.g., Kanai et al., 1989; Edson et al., 1993). That neuronal MAPs do indeed alter microtubule rigidity *in vivo* is suggested by visual inspection of microtubule behavior in living cells: nonstabilized microtubules bend readily (Cassimeris et al., 1988; Sammak and Borisy, 1988), whereas MAP-stabilized microtubules appear much straighter and more resistant to bending even when not bundled (Kaech et al., 1996). In contrast with MAP2 and tau, the microtubule-stabilizing drug taxol, which also induces bundling of microtubules but has no stiffening effect, is unable to induce cell process formation (Edson et al., 1993). Taken together, these observations indicate that the increase in microtubule rigidity induced by tau and MAP2 may be essential for the formation and maintenance of neuronal processes. For this reason, the molecular mechanisms of the effects observed here are well worth studying.

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