Single-molecule investigation of the interference between kinesin, tau and MAP2c

Arne Seitz¹, Hiroaki Kojima²,
Kazuhiro Oiwa², Eva-Maria Mandelkow,
Young-Hwa Song and Eckhard Mandelkow

Max-Planck-Unit for Structural Molecular Biology,
Notkestrasse 85, D-22607 Hamburg, Germany and

²Kansai Advanced Research Center, Communications Research
Laboratory, Kobe 651-2492, Japan

¹Corresponding author
e-mail: seitz@mpasmb.desy.de

Motor proteins and microtubule-associated proteins (MAPs) play important roles in cellular transport, regulation of shape and polarity of cells. While motor proteins generate motility, MAPs are thought to stabilize the microtubule tracks. However, the proteins also interfere with each other, such that MAPs are able to inhibit transport of vesicles and organelles in cells. In order to investigate the mechanism of MAP-motor interference in molecular detail, we have studied single kinesin molecules by total internal reflection fluorescence microscopy in the presence of different neuronal MAPs (tau, MAP2c). The parameters observed included run-length (a measure of processivity), velocity and frequency of attachment. The main effect of MAPs was to reduce the attachment frequency of motors. This effect was dependent on the concentration, the affinity to microtubules and the domain composition of MAPs. In contrast, once attached, the motors did not show a change in speed, nor in their run-length. The results suggest that MAPs can regulate motor activity on the level of initial attachment, but not during motion.

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Introduction

Microtubules (MTs) are involved in diverse cellular processes, e.g. cell morphogenesis, cell division and intracellular trafficking. In cells, MTs can serve as tracks for organelle transport mediated by MT-dependent motor proteins, such as the plus end-directed kinesin or the minus end-directed motor dynein. These motors (for kinesin reviews, see Vale and Milligan, 2000; Woehlke and Schliwa, 2000) can transport their cargoes towards the cell periphery or back towards the MT organizing centre (MTOC).

MTs are decorated with MT-associated proteins such as tau, MAP2 or MAP4, which promote MT assembly (Hirokawa, 1994). They play important roles in organizing the MT cytoskeleton; in particular, they promote neurite outgrowth and control bundling and spacing of micro-

tubules *in vivo* (Chen *et al.*, 1992). In addition, MAPs can affect the motility of vesicles within cells. For example, excess tau inhibits the plus end-directed transport of vesicles along MTs by kinesin so that the minus end-directed transport becomes more dominant (Ebner *et al.*, 1998). This effect is linked to the attachment and detachment cycle of motors. Intracellular trafficking was biased because tau reduces the attachment of kinesin to MTs, whereas the velocity itself is not affected (Trinczek *et al.*, 1999). In neurons, this leads to a deficiency of vesicles and organelles in cell processes and makes them vulnerable to oxidative stress (Stamer *et al.*, 2002). Analogous phenomena were observed with other MAPs, e.g. MAP4 (Bulinski *et al.*, 1997).

The molecular mechanism of the MAP-motor interference has been a matter of debate (von Massow *et al.*, 1989; Heins *et al.*, 1991; Lopez and Sheetz, 1993; Hagiwara *et al.*, 1994). Together, these results suggested that there is an inhibition of motility caused by fibrous MAPs like tau, MAP2 or MAP4, at least in certain experimental settings as seen by the MT gliding assay. However, due to the nature of the multi-motor assay, the effect of the MAPs on motor processivity could not be deduced from these experiments; in particular, the effect on the run-length remained unknown.

In a simple model of transport and interaction of kinesin with MAPs, one would expect to find a decrease in run-length when the track (MT) is paved with obstacles like MAPs. Our previous results (Trinczek *et al.*, 1999) endorse this picture. We found a decrease in run-length for both kinesin or dynein when MAPs were over-expressed in cells, combined with a decrease in attachment frequency, especially for kinesin. In these studies too, the number of motor molecules responsible for the movement of a vesicle or organelle was undetermined, and therefore it was difficult to draw conclusions for individual motors.

The aim of our present study was to clarify the mechanism underlying the effect of MAPs on the motion of single kinesin molecules, using the method of total internal reflection fluorescence microscopy (TIRFM; Vale *et al.*, 1996; Toomre and Manstein, 2001). In this assay, the binding and movement of single motors could be analysed separately. In a first set of experiments, the effect of the longest tau isoform, httau40, on the motility parameters of kinesin was investigated in detail. Afterwards, the effect of other MAPs and other tau isoforms was investigated as well. These experiments were complemented by ATPase and MT-binding assays in order to correlate the single molecule results with the overall behaviour in solution. The results contribute to a better understanding of the molecular MAP-motor interactions and the implications for the movement of the molecular motor kinesin.

Results

Attachment of MAPs and motors to microtubules

In order to study the MAP-motor interference on the MT surface in a single molecule assay, several non-standard conditions had to be chosen whose applicability had to be ascertained first. The most important constraint is that very low concentrations are required for observing single motor particles on MTs. Thus, MTs are typically decorated with MAPs at initial concentrations around 1 μM tubulin; they are diluted ~10-fold (down to ~0.1 μM) for application to the flow cell, followed by further dilution (down to ~1 nM) by rinsing. For the duration of the experiment, they remain sufficiently stable in the presence of the drug taxol (Caplow *et al.*, 1994). At these low concentrations, attachment rates of motors would become very rare in standard MT reassembly buffers [e.g. 100 mM K^+ -PIPES pH 6.9, 1 mM EGTA, 1 mM MgCl_2 , 1 mM dithiothreitol (DTT), 1 mM GTP, ionic strength ~160 mM] and make it difficult to record statistically significant numbers of events. This can be counteracted by lowering the ionic strength, which increases the attachment rates of motors, but has little influence on the processivity (Vale *et al.*, 1996). A second consideration is that MAPs would be almost fully bound in the micromolar concentration range and in typical reassembly buffers, but not necessarily after dilution (since K_d values are in the 0.1–1 μM range; Goode and Feinstein, 1994; Gustke *et al.*, 1994; Ackmann *et al.*, 2000). Thus, we had to find conditions to ensure that the decoration of MTs by MAPs could be quantified even after dilution. Since the MAP-MT binding is dominated by electrostatic interactions (Vallee, 1982), it was anticipated that low ionic strength would increase the affinity of MAPs, similar to that of motors. To investigate this, a pelleting assay of htau40 was performed in single molecule assay buffer BRB12 (ionic strength ~20 mM), as described by Ackmann *et al.* (2000). In Figure 2A and B, the htau40 concentrations ranged from 3.5 μM (lane 1) to 0.4 μM (lane 8). Only the highest concentrations showed traces of tau in the supernatant, while the bulk of the protein stayed in the MT pellet, even at low concentrations. The control sample (K) contained only htau40 (3.5 μM) in order to ensure that the protein did not aggregate under these conditions. This experiment shows that under the low salt condition, the binding of htau40 is limited by the initial concentration of htau40. Saturation was reached when every tubulin monomer bound one htau40 molecule. These results indicate a strong binding of htau40 to MTs under low ionic strength conditions.

To confirm that MAPs are not detached after the dilution in single molecule assay buffer, the following control experiments were performed. MTs (~2 μM) were incubated with green fluorescent protein (GFP)-htau40 (~5 μM) for 20 min, centrifuged and resuspended in assay buffer so that a tubulin concentration of 0.1 mg/ml (~1 μM) was obtained. This was the stock solution for a dilution series in single molecule assay buffer. The concentration of MT in the TIRFM experiment was estimated to be 1 nM. To obtain comparable MT concentrations, we diluted the prepared stock solution 1000-fold. The amount of GFP-htau40 bound to the MT was measured by fluorescence microscopy. Figure 2C shows the intensity profile of a MT along its length, and the specific fluorescence for different

dilutions (Figure 2D), calculated from at least 20 different MTs. The results show that dilution of the MAP-decorated MTs in the low ionic strength conditions of the single molecule assay does not lead to a displacement of the MAPs from the MTs (in contrast to normal reassembly buffer, where tau largely dissociated from MTs at low concentrations; data not shown).

Effect of htau40 on the motility of kinesin (rk555-GFP) in the single molecule assay measured by TIRFM

Our first approach to determine the influence of MAPs on the motility of kinesin was to investigate the effect of the longest tau isoform htau40 on the motility of kinesin (rk555-GFP). Polymerized MTs (~1 μM) were incubated with different amounts of htau40 (0–4 μM). After dilution (10-fold) in assay buffer, we performed a single molecule experiment as described in Materials and methods. An example of kinesin (green/yellow) moving smoothly with no stops along a MT (red) is shown in Figure 1B. As shown above, the dilution does not lead to the displacement of MAPs from the MT.

Next we studied the effect of htau40 on the run-length of kinesin. In Figure 3, the determination of the run-length of kinesin (rk555-GFP) is shown for four different ratios of htau40 and tubulin dimer concentration (molar ratio htau40:MT = 0, 0.5, 1.0 and 1.5). The run-lengths of 100–200 events per experiment were measured manually with an image processor (ARGUS20, Hamamatsu) and binned in 0.6–0.9 μm intervals. This procedure leads to an exponential distribution (Block *et al.*, 1990; Vale *et al.*, 1996), and the exponential decay constant is a measure of the mean run-length. In all four cases, a good correlation between the run-length distribution and the fitted curve was obtained. This is corroborated by the agreement between the run-length obtained with the fitting procedure and by calculating the arithmetic mean value (Table I). Increasing htau40 concentrations between 0 and 1.5 μM did not lead to a change in run-length (Figure 4A), which remained constant at ~1.8 μm . This value is in good agreement with the run-length reported by other groups (e.g. 1.5 μM , Thorn *et al.*, 2000; 1.8 μm , Tomishige and Vale, 2000) for the corresponding human kinesin construct (hK560-GFP). It was not necessary to correct the obtained run-length for the influence of photobleaching, because the bleaching rate was 10-fold lower than the dwell time of a kinesin molecule on the MT (see Materials and methods).

In contrast to run lengths, the attachment rates of kinesin decreased dramatically at higher concentrations of htau40 (htau40:MT > 1.5), so that the number of events was too low to perform a reliable fit to the distribution (Figure 4B; Table III). At high concentrations of htau40 (htau40:MT > 3) hardly any movement was detected. This means that the crucial step of movement is the attachment of kinesin to the MTs. Once it is on the track it moves with an intrinsic velocity that does not depend on tau.

The velocity of the molecular motor is directly coupled to the catalytic cycle of the protein and is another important parameter, besides run-length and binding frequency. The velocities of the motor for the four different htau40 concentrations are summarized in Table II and Figure 4C. Mean velocities were calculated using two methods: (i) for each sample, the mean velocity (total distance/total time)

Fig. 1. (A) Domain structure of MAPs. ht40 is the longest isoform in the human CNS, occurring mostly in axons. It consists of 441 amino acid residues and can be divided into an N-terminal projection domain and a C-terminal MT-binding domain containing four imperfect repeats (R1–R4) and basic proline-rich sequences on either side that act as MT-targeting domains. The repeats show high homology between tau, MAP2 and MAP4. The inserts near the N-terminus and the second repeat R2 can be alternatively spliced, resulting in six tau isoforms. K23 is a tau construct containing all of ht40 except the repeats. It binds to MT, but unproductively, i.e. it does not stabilize MT. (B) Kinesin moving along MT. Six snapshots of a real-time (30 Hz) video sequence (four frames rolling averaged) showing kinesin (rK555–GFP) moving smoothly along the MT (labelled with Cy3) with a constant speed. The images from the two different fluorescence channels are superimposed. The MT is shown in red and kinesin in green.

was calculated; and (ii) the velocities were calculated for all events separately, binned in intervals between 0.05 and 0.1 $\mu\text{m/s}$, and the mean velocity was determined by a Gaussian fit to this distribution. Both methods led to the same results. The mean velocities were ~ 0.4 – 0.5 $\mu\text{m/s}$, comparable with the values of other groups for recombinant brain kinesin (e.g. 0.37 $\mu\text{m/s}$, Thorn *et al.*, 2000; 0.32 $\mu\text{m/s}$, Romberg *et al.*, 1998). More importantly, the mean velocity did not change when ht40 was present on the MT, consistent with other *in vitro* studies on MT gliding in the presence of MAPs (Paschal *et al.*, 1989; Heins *et al.*, 1991; Lopez and Sheetz, 1993).

ATPase assay for kinesin as a function of the ht40 concentration

To investigate the influence of MAPs on the ATPase of the molecular motor, a MT-activated ATPase assay for

Fig. 2. Binding of ht40 to MTs at low concentration and ionic strength. (A and B) MTs (initial concentration 0.9 μM) were incubated with ht40 (initial concentrations ranging from 0.4 to 3.5 μM) in low salt conditions (ionic strength 20 mM). (A) Horizontal slices of two 10% SDS gels of the supernatant (S) and the pellet (P) at initial concentrations. On the right, three calibration samples of ht40 (0.3, 0.6 and 0.9 μg ht40) are shown as well. In K, a control sample of ht40 is shown to ensure that the protein is not aggregated during the binding assay. Most tau was bound to MTs, therefore only the two highest concentrations show traces in the supernatant. Decreasing amounts of ht40 were found in the pellet (lanes 1–8), correlating with the decrease of the initial ht40 concentration. (B) Concentrations of ht40 in the supernatant (bottom curve) and pellet (top curve) are plotted against the total initial concentration of ht40. (C and D) MTs (2 μM , stabilized with 20 μM taxol) were decorated with GFP-ht40 (5 μM), diluted up to 1000-fold (down to 1 nM) using low-salt buffer, and the fluorescence was measured with an inverted microscope. (C) Typical line-scan along a MT, showing that the specific labelling by GFP-tau is roughly constant along the length. (D) Specific fluorescence intensities of decorated MT after dilution. There is no change in fluorescence intensity, indicating that dilution does not lead to the displacement of ht40 from MTs.

Fig. 3. Distribution of travel distances of kinesin (rK555-GFP) along microtubules in the presence of tau (htau40 isoform). The histograms show travel distances binned in 0.6–0.9 μm intervals. The exponential decay constant fitted to this distribution is defined as the mean run-length of the examined molecular motor. (A–D) Results for increasing htau40 concentration (tau:tubulin ratios = 0, 0.5, 1.0 and 1.5). The distributions are similar, indicating that the run-length does not change in these conditions, in contrast to the attachment frequency (see text).

rK555-GFP in the presence of htau40 was performed in the same buffer conditions as the motility assay. The polymerized and taxol-stabilized MTs were incubated with different amounts of htau40 for 15 min at 37°C in assay buffer BRB12 including 1 mM of ATP, and then the ATPase assay was performed. The ATP consumption per second and kinesin head was measured at different MT concentrations (0–7 μM) and can be described by Michaelis–Menten kinetics, yielding V_{max} and $K_{\text{M}}(\text{MT})$. The apparent $K_{\text{M}}(\text{MT})$ reflects an affinity of kinesin for the MT, its allosteric activator. The maximal ATP turnover rate V_{max} is determined by the rate-limiting step in the catalytic cycle (Woehlke *et al.*, 1997). Figure 5A shows five curves, each of which represents one dataset for a constant tau:tubulin ratio between 0 and 1.3, comparable with the ratios used in the single molecule experiment. For undecorated MT, we found $K_{\text{M}} = 2.8 \mu\text{M}$ and $V_{\text{max}} = 18.8 \text{ P}_i/(\text{head} \cdot \text{s})$ for kinesin (rK555-GFP). These values are in good agreement with the values reported from other groups for the corresponding human kinesin construct (hK560; Moyer *et al.*, 1998; Romberg *et al.*, 1998; Brendza *et al.*, 2000). When MTs were decorated with htau40, the ATP consumption decreased in a dose-dependent manner. Figure 5B shows $K_{\text{M}}(\text{MT})$ and V_{max} values obtained from a fit to the data of Figure 5A, plotted against the tau:tubulin ratio. No change in V_{max} (circles) was observed with increasing MAP concentration on the MT (top line). In contrast, there is a steep dependence of $K_{\text{M}}(\text{MT})$ and the concentration of htau40. This suggests that the affinity of kinesin is lowered when the MTs are decorated with htau40, presumably because kinesin and MAPs compete for the same or overlapping binding place (competitive inhibition). The dependence of $K_{\text{M}}(\text{MT})$ on the concentration of the inhibitor (htau40) is not linear, as would be expected for the simplest (linear) competitive inhibition, but has a parabolic shape, indicating a more complex interplay between ATPase, motor movement and tau. For example, the observed shape could be explained if

Table I. Run-length (μm) for rK555-GFP in a single molecule assay

$c_{\text{htau40}}/c_{\text{MT}}$	n	Mean run-length (μm)	SD (μm)	SE (μm)	λ (μm)	$\Delta\lambda$ (μm)
0	141	1.45	1.17	0.10	1.78	0.16
0.5	158	1.92	1.52	0.11	1.85	0.14
1	149	1.63	1.40	0.12	1.59	0.13
1.5	156	1.61	1.44	0.12	1.89	0.17

Single molecule measurements were performed as described in Materials and methods. n , number of events scored for each htau40 concentration. Mean run-length is the arithmetically averaged run-length, SD is the standard deviation and SE is the standard error. λ is the decay constant of the exponential curve fitted to the distribution of run-lengths.

$n(x) = \exp(-x/\lambda)$ is the number of events in a given run-length bin, where x is the run-length and $\Delta\lambda$ is the error of λ given by the fit.

two molecules of htau40 were necessary to inhibit the docking of kinesin completely (Rode *et al.*, 1984).

When comparing the ATPase results with the single molecule data, we noted a correlation between the attachment rate (Figure 4B) and $K_{\text{M}}(\text{MT})$ (Figure 5B), both of which depend on the ratio htau40:tubulin. This suggests that the rise in K_{M} reflects the decrease in kinesin's attachment frequency. On the other hand, neither the maximal ATPase nor the instantaneous velocity depend on tau, presumably reflecting the fact that once the motors are in the state of movement they consume ATP at their intrinsic pace.

Effect of different MAPs on the motility of kinesin measured by the single molecule assay

To get a better impression of the mechanism of the MAP-motor interaction, we wanted to investigate the effect of other MAPs and tau isoforms as well. We compared juvenile MAP2c (containing three repeats), the longest and the shortest isoform of tau with three repeats (htau39 and htau23 differing by 58 residues in the projection domain), and a tau construct without inserts and repeats (K23), which binds to MTs without stabilizing them (Gustke *et al.*, 1994). In all cases, elevated concentrations of MAPs lowered the attachment rate of kinesin without affecting the velocity, as described above. However, closer inspection shows that the proteins fall into two categories. The first comprises MAP2c and htau23, whose effects are comparable with that of htau40 (Figure 6A). These three proteins cause a smooth decrease of attachment frequency; they differ only in the concentration needed to inhibit movement of kinesin (half-maximal inhibition at MAP:tubulin ratios of 0.18 for MAP2c, 0.51 for htau23 and 2.21 for htau40). MAP2c is the most effective inhibitor of attachment, in agreement with its strong binding to MTs (Wille *et al.*, 1992), followed by htau23. Unexpectedly, the longest splice isoform, htau40, was less effective than htau23, in spite of its tighter binding to MTs.

The second group contained htau39 and K23 (Figure 6B). As before, at high concentrations, a decrease of the attachment frequency of kinesin was found, but at lower concentrations, these two proteins appeared to facilitate the attachment rate of kinesin. In other words, a MT becomes $\sim 30\%$ more attractive for kinesin when it is

Fig. 4. Effect of htau40 on the movement of kinesin (rK555-GFP) along the microtubule. (A) The run-lengths determined as shown in the diagram above are plotted against the htau40:tubulin dimer ratio. No change in run-length could be detected when the amount of htau40 on the MT was increased, i.e. the processivity of the motor kinesin is not affected by htau40. (B) In contrast, a dose-dependent effect of the attachment frequency is found. The number of motor molecules interacting with the MT was divided by the MT length, kinesin concentration and the observation time. Without MAPs, the attachment rate is ~ 46 (s $\cdot\mu\text{m}\cdot\mu\text{M}$)⁻¹ (=100%). With increasing htau40 concentration, a steady decrease of the binding frequency is found. At the highest htau40 concentration, almost no movement could be detected. (C) Velocity distributions in four experiments at different concentrations of htau40. The peaks are typically ~ 0.4 – 0.5 $\mu\text{m/s}$ with a spread of ~ 0.2 $\mu\text{m/s}$ (full width at half maximum). There is no systematic dependence on tau.

decorated with low amounts of K23 or htau39. A similar effect was observed for the run-length. In the higher range of MAP concentrations (MAP:tubulin >0.2), the run-length is ~ 1.8 μm , similar to that found with the other MAPs. However, at low decoration levels it becomes $\sim 50\%$ higher (Figure 7), ~ 3.0 μm for htau39 and 2.8 μm for K23. Thus, both the processivity and the run-length of kinesin is increased by sparse decoration of the MT with one of these two MAPs.

Table II. Velocity ($\mu\text{m/s}$) for rK555-GFP measured in single molecule assay

$c_{\text{htau40}}/c_{\text{MT}}$	Mean velocity	SD	SE	v_c	σ
0	0.41	0.15	0.02	0.35 ± 0.01	0.19 ± 0.02
0.5	0.44	0.10	0.01	0.44 ± 0.01	0.17 ± 0.02
1	0.45	0.12	0.01	0.41 ± 0.01	0.14 ± 0.02
1.5	0.47	0.11	0.02	0.46 ± 0.01	0.17 ± 0.01

Mean velocity is the arithmetic average of the velocities measured in single molecule assay. SD and SE are standard deviation and standard error. Velocities were binned (0.05–0.1 $\mu\text{m/s}$ intervals) and v_c (center of the distribution) was determined by a Gaussian fit $[n(v) = e^{-\frac{(v-v_c)^2}{\sigma^2}}]$ to this distribution; where $n(v)$ is the number of events in a velocity bin, v the velocity and σ the width of the distribution.

Table III. Attachment frequency of rK555-GFP measured in single molecule assay

$c_{\text{htau40}}/c_{\text{MT}}$	Attachment frequency (s $\cdot\mu\text{m}\cdot\mu\text{M}$) ⁻¹	Relative frequency	Δ relative frequency
0	46.4	1	0.081
0.5	41.3	0.89	0.079
1	38.1	0.82	0.106
1.5	32.0	0.69	0.080
2	26.6	0.57	0.098
3	13.8	0.30	0.132
4	9.43	0.20	0.180

The kinesin–MT attachment events were normalized with respect to MT length (μm), kinesin concentration (μM) and the observation time (s).

Discussion

Motor-dependent transport along MTs takes place with remarkable efficiency despite the crowded environment within cells. Over the past decade, many groups have contributed to a molecular understanding of this process (e.g. Johnson and Gilbert, 1995; Hancock and Howard, 1999; Vale and Milligan, 2000; Woehlke and Schliwa, 2000). For example, much effort has been spent on the issue of processivity, which is observed only with certain motors (such as conventional kinesin) and has been explained in terms of a hand-over-hand movement of a double-headed motor complex. But the existence of single-headed semi-processive motors has called this into question (Okada and Hirokawa, 2000), and the issue may not matter for the cell when cargoes are carried by several motor complexes simultaneously [see also the recent report that clustering of motors is important for transport (Klopfenstein *et al.*, 2002) and can determine its characteristics (Higuchi and Endow, 2002)]. The question concerning us here is that motors must move along MT tracks covered with MAPs, which are necessary as MT stabilizers (‘ties’), and the competing requirements for dynamics and stability represent an obvious dilemma.

In the past, we and others (von Massow *et al.*, 1989; Lopez and Sheetz, 1993; Hagiwara *et al.*, 1994) studied the ternary system of motors–MAPs–MTs *in vitro*, asking how MAPs might affect motor speed, run-length or attachment, but the results remained ambiguous. The interpretation of the *in vitro* results suffered from the limitations of the

Fig. 5. MT-stimulated ATPase assay of kinesin. (A) MT-stimulated malachite green ATPase assays were performed with different ratios between htau40 and tubulin-dimer (0, 0.3, 0.7, 1.0, 1.3). The data points are fitted to Michaelis-Menten kinetics. (B) $K_M(MT)$ and V_{max} values were plotted against the ratio of htau40 and tubulin-dimer concentration. The V_{max} values do not change with increasing amounts of htau40 on the MT, but a steep increase was observed for $K_M(MT)$.

experimental system, in most cases the MT gliding assay. It is based on many motors that act in concert and would presumably be capable of overriding the local resistance of MAPs. As a consequence, we have now turned to recently developed assay systems involving only single motor proteins. A second motivation for this study was the observation that in cells, different tau proteins are able to inhibit motor-dependent organelle transport (predominantly kinesin-dependent transport), even though they had no visible effect in the MT gliding assay (Ebner *et al.*, 1998). Quantification of vesicle and organelle motility in cells revealed that the run-length and attachment rates were reduced by MAPs, but not the velocity (Trinczek *et al.*, 1999). However, the cellular events are presumably also caused by multiple motors, and therefore we wanted to reduce the complexity by an assay involving only single motors, single MT tracks, and a defined stoichiometry of MAPs.

In order to perform the experiments, it was necessary to adapt the biochemical parameters, in particular the ionic strength and protein concentrations, and to check whether they are justified. The cellular milieu has an ionic strength of ~150 mM, which corresponds closely to the typical MT reassembly buffers *in vitro* (e.g. 100 mM PIPES pH 6.9, ionic strength ~160 mM). At this value, the interactions between kinesin and MTs are too rare to collect data with sufficient statistical significance (at the required low concentrations). To circumvent this, the ionic strength has to be decreased, usually to 20 mM. This appears to be

Fig. 6. Effect of different MAPs on the binding frequency of kinesin (rK555-GFP). The binding frequency of kinesin was measured for different MAPs. (A) Comparison of htau40 (diamonds), MAP2c (triangles) and htau23 (inverted triangles). The effect of the three proteins is very similar. The binding frequency of kinesin decreased monotonically with increasing MAP concentration. The three MAPs only differ in the effective concentration, which is needed to inhibit the movement (half values at MAP:tubulin values of 0.18, 0.51 and 2.21). (B) Results for htau39 (square) and the tau construct K23 (circle) are shown. An activation of the attachment rate beyond that of undecorated MTs is observed for these two proteins at low MAP concentrations. At high concentrations, their effect is comparable with the other MAPs, i.e. the binding frequency decreased monotonically.

Fig. 7. Run-length of kinesin at low concentration of htau39 and K23. Run-length distributions are plotted for the two MAPs, which activated the kinesin attachment rate to MT at low concentration. The exponential fit lead to decay constants of 3.0 μm for htau39 and 2.8 μm for K23.

justified since the processivity is hardly altered (Vale *et al.*, 1996). Secondly, concentrations of MTs and kinesin have to be very low in order to make individual interactions observable. In these conditions, MAPs would mostly dissociate in normal reassembly buffer so that their interference with motors would not be measurable. Fortunately, lowering the ionic strength solves this problem as well. The binding strength of MAPs to MTs increases strongly so that they remain fully bound even after dilution (Figure 2). With these adaptations, the results can be summarized as follows. (i) Once a motor protein has attached to a MT, it moves with a constant speed of $\sim 0.4 \mu\text{m/s}$ (Table II), a typical value for *in vitro* assays of kinesin (Romberg *et al.*, 1998; Thorn *et al.*, 2000). The speed is not influenced by MAPs, even at high MAP:tubulin ratios. The probability of detachment is constant along the path, leading to an exponential decay in the run length distribution (Figure 3). (ii) The run-length, a measure of processivity, is $\sim 1.8 \mu\text{m}$ (~ 225 steps at 8 nm) and also does not depend on MAPs (Figure 4A). (iii) The attachment rate, typically $46 (\text{s} \cdot \mu\text{m} \cdot \mu\text{M})^{-1}$ without MAPs in our standard conditions, steeply decreases when MAPs are present. Half inhibition occurred at a MAP:tubulin ratio of 2.2 (Figure 4B). (iv) The MT-activated ATPase of kinesin shows the usual Michaelis–Menten kinetics, with a V_{max} of $\sim 19 \text{ s}^{-1}$ and a K_{M} of $\sim 2.8 \mu\text{M}^{-1}$ in the absence of MAPs. Whereas V_{max} remains independent of MAPs, K_{M} increases strongly, indicating a lower ‘affinity’. This behaviour is consistent with the reduced attachment rate and can be broadly described as competitive inhibition (Figure 5). (v) The effects of different MAPs are generally similar in that they have no influence on kinesin’s velocity or run-length, but they inhibit the attachment rate in the higher concentration range. This inhibition is not coupled to the MT assembly properties of MAPs since inhibition is also observed with K23, a construct which binds to MTs but does not promote MT assembly. However, the efficiency of MAPs varies (half inhibition at MAP:tubulin ratios between 0.2 for MAP2c and 2.2 for htau40). Two of the MAPs (htau39, K23) are unusual in that they ‘stimulate’ MTs at low occupancy, i.e. attachment is facilitated by $\sim 30\%$ and run-lengths increase by $\sim 60\%$ (Figures 6 and 7). Although the effects are reproducible, their magnitude was not dramatic, and therefore should not be overemphasized.

The interpretation of the first two points is straightforward: when a kinesin molecule is attached and moving along a MT, MAPs do not apparently represent serious obstacles (illustrated in Figure 8C). This could be rationalized by noting that even when MAPs have high affinities, they still have high on- and off-rates (Olmsted *et al.*, 1989), so that they could easily be pushed out of the way by a firmly bound motor. Points (iii) and (iv) mean that MAPs are obstacles only for the attachment of motors, i.e. before the motors are firmly bound (Figure 8A). This would be consistent with the electrostatic nature of the kinesin–MT interaction, which guides kinesin into the proper docking position on a free site (Figure 8B; Thorn *et al.*, 2000; Wang and Sheetz, 2000). In this scenario, the positive charges of MAPs would cover up the MT surface necessary for kinesin’s attachment, leading both to a lower attachment rate and a higher K_{M} value of the MT-activated ATPase. Indeed, docking of the kinesin crystal structure

Fig. 8. Model of kinesin motility on a MAP-decorated microtubule. Kinesin molecules approaching a microtubule protofilament are shown in three states. (A) Kinesin approaching the MT at a site occupied by a MAP is prevented from attachment. (B) Kinesin approaching a free site can dock into starting position on the MT and get ready to move (aided by tubulin’s acidic C-terminus (Thorn *et al.*, 2000)). (C) Once kinesin is properly attached, it can move along the MT without perturbation by MAPs.

onto the MT surface suggests an interaction between kinesin’s neck and tubulin’s C-terminus, where MAPs are thought to bind as well (Kikkawa *et al.*, 2001; Song *et al.*, 2001; see www.mpasmb-hamburg.mpg.de). The capability of some MAPs to facilitate kinesin attachment at low occupancy might be explained by a guidance effect analogous to that of tubulin’s C-terminus.

Some of the above predictions should be testable by comparing different MAPs or MAP constructs. We had expected that the inhibitory effect of MAPs would simply correlate with the binding affinity, but the relationships are more complex. MAP2c binds much more tightly than any tau isoform (Wille *et al.*, 1992), and indeed it is the strongest attachment inhibitor (Figure 6A). In contrast, the adult tau isoform htau40 binds MTs more strongly than the fetal form htau23 (Gustke *et al.*, 1994), and yet htau40 is less inhibitory for kinesin’s attachment. In searching for alternative explanations, we note that the strong inhibitors, MAP2c and htau23, have a higher overall positive charge than htau40 (+10, +10 and +2, respectively; Table IV, column 5), but this argument, based on charge, breaks down for htau39 and K23 (overall charges -1 and $+4$). It is conceivable that a more detailed comparison of the charge distributions might explain the strength of inhibition, but this is not meaningful as long as structural information on the MAPs is lacking. Moreover, since we are dealing with a ternary system (MT–MAPs–motors), it is possible that MAPs prevent the attachment of motors by a direct interaction, independently of blocking binding sites on MTs. This would be akin to an inactive folded conformation of kinesin (Stock *et al.*, 1999). In this context, it is remarkable that htau39 and K23 at low concentrations are capable of stimulating kinesin for attachment and processivity (Figure 6B). In the case of htau39, this is the only tau isoform with a negative net charge (or a slightly acidic pI). This might make the MT more attractive for the positively charged patches on kinesin and thus explain the activation of the MT at low decoration levels.

One of the aims of this work was to overcome some of the technical limitations of previous approaches to studying the interference between MAPs and motors in MT-based transport. Here, we have to distinguish between *in vitro* assays and cell-based assays. The *in vitro* studies

Table IV. Charge distribution and theoretical pI of different MAPs

	Projection domain	Assembly domain	Acidic domain	Basic domain	Total charge	pI
htau40	-15	+17	-24	+26	+2	8.24
htau39	-15	+24	-24	+23	-1	6.85
htau23	-4	+24	-13	+23	+10	9.39
K23	-4	+8	-13	+17	+4	9.08
MAP2c	-16	+26	-18	+28	+10	9.4

Arginine and lysine were regarded as negatively charged, aspartic acid and glutamic acid as positively charged. Histidine was not counted, but is considered in the calculated pI value.

were all performed with the gliding assay where multiple motors carry a MT over a surface (von Massow *et al.*, 1989; Heins *et al.*, 1991; Lopez and Sheetz, 1993; Hagiwara *et al.*, 1994). As might be expected, the inhibitory effect of MAPs is less noticeable in the case of many cooperating motors than in the case of a single motor. This probably explains in part why the published reports vary considerably in terms of the observed inhibition; in addition, the experimental conditions are themselves a source of variability. Nevertheless, there seems to be a consensus that MAPs tend to reduce the number of MTs interacting with the kinesin-coated surface, even though the gliding speed itself is less affected, and the inhibition is greatest with large MAPs (e.g. MAP2 and MAP4). This would be consistent with our observation that MAPs primarily lower the attachment rate of single motors, without affecting the speed itself. No information on run-length was available for the gliding assays, due to the overlapping action of many motors.

In the case of cell-based assays, some authors focused on the general mobility of vesicles (i.e. fraction of moving particles), which was decreased by MAPs, without analyzing speed or run-length in detail (Sato-Harada *et al.*, 1996; Bulinski *et al.*, 1997). This would also be consistent with the reduced attachment rates noted in our present experiments. On the other hand, one of our previous studies (Trinczek *et al.*, 1999) concluded that the run-length of vesicles in CHO cells was decreased by elevated MAPs (from 3 to 1.7 μm), but not the speed. The change in run length is different to what we find here and deserves special comment. In the cellular system, this parameter could only be measured by making constraints (e.g. only runs with lengths >1 μm and velocities >0.3 $\mu\text{m/s}$ were counted), which will affect the result itself. Additionally, the ionic strength in cells is ~150 mM, whereas our assay required low ionic strength. Since both kinesin and MAP interactions with MT depend on the salt concentration, it is possible that the balance between MAP and motor binding is different in cells than in our experiments so that MAPs could represent more effective obstacles. Furthermore, due to the higher viscosity in the cell, the load on the motor would increase and thus reduce its efficiency (Coppin *et al.*, 1997). But the situation becomes even more complicated when we consider that, *in vivo*, more than one motor is responsible for the transport of the organelles. At best, these are identical motors moving the cargo in the same direction, but the possibility of several distinct motors has to be considered (Goldstein, 2001). Indeed, the reversal of direction (from

dynein-based to kinesin-based transport) is particularly sensitive to MAPs (Trinczek *et al.*, 1999) and causes an inward bias of transport, which leads to a clustering of mitochondria and other cell structures around the MTOC. The longer run-lengths seen in cells could result from several motors with their own attachment and detachment rates (see Supplementary figure 2, available at *The EMBO Journal* Online). In this case, a reduction of attachment by elevated MAPs would explain the observed shorter run-lengths. Finally, cellular motors are regulated by motor conformation, adaptors or other protein cofactors, which are controlled in turn by phosphorylation (Morfini *et al.*, 2001). Since the single molecule assay shows little influence of MAPs on run-length, an interesting consequence would be that in the cellular environment MAPs might interfere with the control machinery.

In conclusion, with the single motor assay, it is possible to investigate the molecular mechanism of the MAP-motor interference in a precisely defined *in vitro* system. This has led to a more detailed molecular understanding of how kinesin motility is influenced by MAPs. Since MAPs are linked to transport deficits in cells, these data help to explain MAP malfunctions *in vivo*.

Materials and methods

Preparation of kinesin

A construct containing the 555 N-terminal amino-acids of rat kinesin were C-terminal coupled to EGFP (from pEGFP-N1; Clontech) and cloned into a pET21a vector (Novagen). This construct (rK555-GFP), comprising the motor domain and part of the stalk domain of conventional kinesin from rat brain, is equivalent to the hK560 construct from human conventional kinesin used in earlier studies by Vale and co-workers (Woehlke *et al.*, 1997; Case *et al.*, 2000; Thorn *et al.*, 2000). The vector of rK555-GFP was transformed into *Escherichia coli* BL21(DE3)RIL, grown in Luria-Bertani medium at 37°C to an OD₆₀₀ of 0.6–0.8, and protein expression induced for 24 h at 24°C with 0.1 mM IPTG. Cells were lysed in a French press in buffer A [20 mM K⁺-PIPES pH 6.9, 1 mM MgCl₂, 1 mM EGTA, 1 mM DTT, 150 mM NaCl, 10% (v/v) glycerol] containing 1 mM PMSF and 1 mM benzamidine. After centrifugation (28 000 g), the supernatant was incubated with phosphocellulose (Whatman P11) for 20 min at 4°C, centrifuged, washed with buffer A and eluted with 250 mM NaCl in a batch treatment. After desalting on a fast desalting column (G25; Amersham Biosciences), the protein was further purified by Mono-Q chromatography using a 0.15–1 M NaCl gradient in 20 mM PIPES [pH 7.5, 1 mM MgCl₂, 1 mM EGTA, 1 mM DTT, 150 mM NaCl, 10% (v/v) glycerol]. The protein eluted at 0.4 M NaCl. The protein was then subjected to a microtubule affinity purification step by incubating it with MTs stabilized with 10 μM taxol (paclitaxel; Sigma) and 1 mM AMP-PNP. After centrifugation, the motor protein was released from the MT with 5 mM MgATP/200 mM KCl. Protein concentrations were determined by Coomassie Blue staining using bovine serum albumin (BSA) as standard (Bioquant, Merck).

Preparation of MAPs

To investigate the MAP–motor interference, we used the largest tau isoform (htau40, 441 residues, containing all four repeats in the C-terminal half and both inserts near the N-terminus), the largest (htau39) and the shortest (htau23) three repeat isoform and a tau construct K23 without the repeats and inserts (Figure 1). Besides these tau constructs/isoforms, the juvenile MAP2c was also used to decorate MTs. The bacterial expression, purification, and binding to MTs of these proteins were reported previously (Gustke *et al.*, 1994). Protein concentrations were measured spectroscopically using the absorption of the aromatic amino acids at 276 nm. The molar dekadic absorption coefficients were calculated theoretically using the known amino acid composition of the proteins. They are as follows: htau40 $\epsilon(276 \text{ nm}) = 7395 \text{ mol}/(\text{g} \times \text{cm})$; htau39 $\epsilon(276 \text{ nm}) = 7250 \text{ mol}/(\text{g} \times \text{cm})$; htau23 $\epsilon(276 \text{ nm}) = 7250 \text{ mol}/(\text{g} \times \text{cm})$; MAP2c $\epsilon(276 \text{ nm}) = 12650 \text{ mol}/(\text{g} \times \text{cm})$; K23 $\epsilon(276 \text{ nm}) = 7250 \text{ mol}/(\text{g} \times \text{cm})$.

Single molecule experiment

The experimental setup of the TIRFM (see Supplementary figure 1) at KARC is described in Oiwa *et al.* (2000). The beam of a laser (488 nm, argon ion laser, Melles Griot 532-A-A03) was expanded and circularly polarized by passing through a quarter-wave plate. It was focused with a lens onto a trapezoidal prism (fused silica), which sat on top of a flowcell. This flowcell was formed by two coverslips with 50 μm polycarbonate spacers between them. The lower glass coverslip was 120–170 μm thick, and the upper surface was formed by a fused silica coverslip (350 μm thick). The gap between the prism and the flowcell was filled with fluorescence-free pure glycerol. The illuminated area was $\sim 20 \times 20 \mu\text{m}$ (corresponding volume = 20 nl). Due to the angle of the beam relative to the flowcell, it is possible to adjust total internal reflection conditions. The fluorescent molecules were visualized with an inverted microscope using an objective (Plan-Apo $\times 100$, n.a. = 1.4; Olympus), the corresponding filter set (for GFP: Olympus U-MWIBA/GFP; for Cy3: Olympus U-MWIB/GFP) and an image intensified CCD camera (C2400; Hamamatsu), which was coupled to another image-intensifier (VS4-1845, Videoscope). The images were recorded on a S-VHS videotape with a frame rate of 30 Hz. In addition to the described reflection-type setup, an objective-type setup was used as well (Tokunaga *et al.*, 1997). The advantage of this latter setup is that ordinary coverslips and immersion oil could be used.

For the single-molecule fluorescent motility assay, rK555–GFP was diluted to a concentration of 1–50 nM (chosen to optimize the detectability of motion events against the background of non-motile molecules) in 12 mM K–PIPES buffer pH 6.9 that additionally contained 1 mM EGTA and 2 mM MgCl_2 (=BRB12 buffer). MTs were visualized by labelling with the fluorophore Cy3 (Amersham Biosciences) coupled to random lysines on the MT surface following the procedure of Hyman *et al.* (1991). First 10 μl of Cy3-MT (0.1 μM) in BRB12 were injected into the flowcell. The MTs (stabilized by taxol) were polymerized using unlabelled and Cy3-labelled brain tubulin at a ratio of 10:1. After several minutes, the flowcell was rinsed with 10 μl of BRB12 containing 1 mg/ml casein (to prevent adsorption of kinesin onto the glass surface), taxol (10 μM) and an oxygen scavenger system (4 mg/ml glucose, 100 $\mu\text{g}/\text{ml}$ glucose oxidase, 20 $\mu\text{g}/\text{ml}$ catalase, 1 mM β -mercaptoethanol). After that, 10 μl kinesin in BRB12 containing casein and the oxygen scavenger system, taxol (10 μM) and 1 mM ATP was applied to the flowcell twice.

For analysis of run-length, binding frequency and velocity, four frames of the videotape were averaged with an image processor (ARGUS20, Hamamatsu). Time points and positions of attachment and detachment were determined manually for single fluorescent spots interacting with the MT. These values were then converted into run-length, association time (the time in which a kinesin molecule interacts with the MT) and velocity. The run-length values were binned (typically into 0.5–1 μm intervals) and the number of events was plotted against the run-length. Assuming a constant probability of detachment per step for a processive motor-like kinesin, one expects an exponential decay of the run-length distribution, in agreement with the observations. The decay constant of the exponential curve fitted to this distribution is defined to be the (mean) run-length (Block *et al.*, 1990; Vale *et al.*, 1996). Note that this definition differs from the mean run-length (=arithmetic average) used in our previous analysis of cellular vesicle movements (Trinczek *et al.*, 1999). In the case of a purely exponential decay, the mean run-length equals the reciprocal decay constant. For obtaining the attachment frequency of motor molecules to MTs, every event of a motor settling on a MT was counted and divided by the total observation time, the protein concentration and the MT length (Romberg *et al.*, 1998).

Photobleaching was measured with MTs completely decorated with rK555–GFP kinesin in the presence of the ATP analogue AMP–PNP. The lifetime τ of the rK555–GFP kinesin was determined to be $\tau = 60 \text{ s}$. The probability of photobleaching was estimated with the following equation: $P = 1 - \exp(-t/\tau)$, where t is the dwell time of the kinesin on the microtubule and τ the lifetime of the fluorescent molecule. For a given dwell time of $t = 4 \text{ s}$ on the MT (corresponding to a mean run-length of 1.6 μm and a velocity of 0.4 $\mu\text{m}/\text{s}$), the probability of photobleaching will be 6%. A dwell time of 16 s (mean run-length $\sim 6 \mu\text{m}$) led to bleaching of 23% of the molecules. This shows that the run-length need not be corrected as long as dwell times do not exceed 10 s.

Decoration of microtubules

Preparation of phosphocellulose-purified tubulin (PC tubulin) after a MAP-depleting step and polymerization of MTs have been described previously (Mandelkow *et al.*, 1985). MTs were decorated with MAPs by incubating them at a concentration of 0.1 mg/ml ($\sim 1 \mu\text{M}$ tubulin dimer) with different amounts of MAPs (0.02–5 μM) for 20 min at 37°C in BRB12 including 10 μM taxol (effective ionic strength 20 mM; note that low ionic strength, compared with the more usual 100 mM PIPES buffers, is necessary to ensure efficient attachment of kinesin and MAPs, see Results). All ratios between tubulin dimers and MAPs refer to the mixing stoichiometry. For the single molecule assay, the MAP-decorated MTs were diluted 10-fold, i.e. to 100 nM tubulin and 2–500 nM MAPs. Kinesin concentration was in the range of 1–50 nM.

For quantifying MAPs bound to MTs, taxol-stabilized MTs (0.9 μM) were incubated for 15 min with different amounts of htau40 (~ 3.5 –0.5 μM) in the single molecule assay buffer (BRB12, 20 mM ionic strength). The solution was centrifuged at 100 000 g for 15 min. The supernatant was removed and analysed by 10% SDS–PAGE. The pellet was resuspended in BRB12 buffer with additional 500 mM NaCl to detach MAPs from MTs. After 15 min incubation at 37°C, the MTs were spun down and the supernatant containing htau40 was analysed by 10% SDS–PAGE. Protein bands were quantified by scanning the gel using the TINA 2.01 (Raytest) software.

ATPase assay

The MT-stimulated ATP hydrolysis of kinesin was measured with the modified malachite green method (Kodama *et al.*, 1986). All reactions were performed at 20°C in BRB12 containing additional 1 mM MgATP and 20 μM taxol. The ATPase reaction of kinesin (rK555–GFP) was measured at different MT concentrations (0–7 μM). The reactions were started by adding ATP and stopped after 2 min by adding ice-cold 0.6 M perchloric acid.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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