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An Isoform of Microtubule-associated Protein 4 Inhibits Kinesin-driven Microtubule Gliding

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Recently, we revealed that microtubule-associated protein (MAP) 4 isoforms, which differ in the number of repeat sequences, alter the microtubule surface properties, and we proposed a hypothesis stating that the change in the surface properties may regulate the movements of microtubule motors [Tokuraku *et al.* (2003) *J Biol Chem* 278: 29609–29618]. In this study, we examined whether MAP4 isoforms affect the kinesin motor activity. When the MAP4 isoforms were present in an *in vitro* gliding assay, the five-repeat isoform but not the three- and four-repeat isoforms inhibited the movement of the microtubules in a concentration-dependent manner. The observation of individual microtubules revealed that in the presence of the five-repeat isoform, the microtubules completely stopped their movements or recurrently paused and resumed their movements, with no deceleration in the moving phase. The result can be explained by assuming that kinesin stops its movement when it encounters a microtubular region whose properties are altered by the MAPs. A sedimentation assay demonstrated that the MAP4 isoforms did not compete with kinesin for binding to microtubules, indicating that kinesin can bind to the MAP-bound microtubules, although it cannot move on them.

Key words: kinesin, MAP4, MAPs, microtubule, motor.

Abbreviations: MAPs, microtubule-associated proteins; Pro-rich, proline rich; AP, assembly-promoting; 20 PME, 20 mM Pipes-KOH (pH 6.9), 1 mM $MgCl_2$, 1 mM EGTA; PA_3T, MAP4 fragment containing the Pro-rich region, the Repeat region with three AP sequences, and the Tail region; PA_4T, MAP4 fragment containing the Pro-rich region, the Repeat region with four AP sequences, and the Tail region; PA_5T, MAP4 fragment containing the Pro-rich region, the Repeat region with five AP sequences, and the Tail region.

In eukaryotic cells, the cytoskeleton is essential for complicated and elaborate cellular functions such as intracellular transport, cytoplasmic organization, cell division and muscle contraction (*1*). Diverse cytoskeleton motors that generate movements along the rails of the cytoskeleton by using the chemical energy of ATP hydrolysis play important roles in these events. The cytoskeleton motors are categorized into microtubule motors (kinesin and dynein families) and actin motors (myosin family) based on the difference in their rail structures. The microtubule motors transport vesicles and organelles along microtubule rails in cells (*2*). Since the motor-bound cargoes should move in an orderly fashion along the microtubule rails, it is likely that the microtubule motor system contains some regulatory modules that bind to microtubules and regulate their properties.

Recently, we cloned microtubule-associated protein (MAP) 4 isoforms that are generated by alternative splicing (*3*). MAP4 has a region known as the Repeat region that contains three to five imperfect repeats of the assembly-promoting (AP) sequence, and the only structural difference among the isoforms is the number of repeat sequences. Since the binding of MAP4 altered the microtubule surface properties and the effects of the alteration were found to differ among isoforms, we proposed a hypothesis that the MAP4 isoforms regulate the movement of microtubule motors by binding to microtubules in order to alter their properties (*3*).

In this study, we examined whether the MAP4 isoforms affected the movement of kinesin by an *in vitro* gliding assay that involves the observation of the movement of fluorescent-labelled microtubules on a kinesin-coated glass surface. Although the microtubules could glide on the kinesin-coated glass surface in the presence of the three- or four-repeat isoforms, the gliding was greatly affected when a five-repeat isoform was present. The observation of individual microtubules demonstrated that the five-repeat isoform alone remarkably impeded or inhibited kinesin-driven microtubule gliding. It is likely that the MAP4 isoform plays an important role as a rail-binding regulator of the microtubule motor system.

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MATERIALS AND METHODS

Purification of the Proteins—Tubulin was prepared from the porcine brain by the standard method (*4*, *5*), and it was labelled with rhodamine (*6*). The expressions and purifications of the microtubule-binding domain fragments of MAP4 isoforms [MAP4 fragment containing the Pro-rich region, the Repeat region with three AP sequences, and the Tail region (PA_3T), MAP4 fragment containing the Pro-rich region, the Repeat region with four AP sequences, and the Tail region (PA_4T) and MAP4 fragment containing the Pro-rich region, the Repeat region with five AP sequences, and the Tail region (PA_5T)] were carried out as described previously (*3*). The human kinesin-1 fragment (1–560 residues) was purified from *Escherichia coli* cells expressing the recombinant protein (*7*). The protein concentration was estimated by the method of Lowry *et al*. (*8*) using bovine albumin as the standard. SDS-PAGE was carried out according to the method described by Laemmli (*9*) using a 10% gel.

Gliding Assay—Gliding assays were performed using rhodamine-labelled microtubules and a fluorescence microscope (Eclipse E600; Nikon, Tokyo, Japan) according to the standard method (*10*), with some modifications. Rhodamine-labelled tubulin was polymerized in advance in 20 mM Pipes-KOH (pH 6.9), 1 mM $MgCl_2$ and 1 mM EGTA (20 PME) containing 10 μM taxol and 100 mM KCl at 37°C for 30 min. A kinesin solution (0.35 μM kinesin and 0.2 mg/ml casein in 20 PME) was introduced into flow chambers that consisted of a glass slide, a pair of spacers and a coverslip. After incubation for 1 min at 21°C, a casein solution {0.5 mg/ml casein and 100 mM KCl in 20 PME [20 mM pipes-KOH (pH 6.9), 1 mM $MgCl_2$, 1 mM EGTA]} was perfused, and the chambers were further incubated for 0.5 min at 21°C. Next, a microtubule solution (0.16 μM rhodamine-labelled microtubule and 10 μM taxol in casein solution) was perfused. After incubation for 2 min at 21°C, an ATP solution containing the MAP4 isoform (1 mM ATP, 8 μg/ml catalase, 20 mM glucose, 20 μg/ml glucose oxidase, 1% mercaptoethanol and 0.5% methylcellulose in casein solution containing various concentrations of the MAP4 isoforms) was perfused to initiate the motor reaction. The microtubule movements were observed at 21°C.

Sedimentation Assay—Tubulin was polymerized in 80 mM Pipes-KOH (pH 6.9), 1 mM $MgCl_2$, 1 mM EGTA and 10 μM taxol at 37°C for 30 min. The microtubule solution was mixed with kinesin and MAP4 isoforms [final condition: various concentrations of the MAP4 isoforms, 2 μM kinesin, 5 μM tubulin, 10 μM taxol, 5 mM MES-KOH, 20 PME, 100 mM KCl, 0.2 mg/ml casein, 8 μg/ml catalase, 20 mM glucose, 20 μg/ml glucose oxidase, 1% mercaptoethanol and 1 mM ATP (pH 6.9)]. The mixtures (30 μl) were incubated at 25°C for 15 min, and they were centrifuged at 24000 × *g* for 60 min at 25°C. The precipitates were resuspended in 30 μl of a 20 PME buffer containing 100 mM KCl, and the supernatants and precipitates were subjected to SDS-PAGE.

RESULTS

Protein Preparations—In this study, an *in vitro* gliding assay (*10*) was used to assess the effects of the MAP4 isoforms on kinesin activity. Microtubules assembled from purified tubulin and intact MAP4 have 25-nm long lateral projections that correspond to the projection domains of MAP4 (*11*, *12*). Since the length of the lateral projection is longer than that of the kinesin motor domain, it is likely that the interaction between the projection domain and the glass surface interferes with the accurate assessment of the motility. Therefore, we used microtubule-binding domain fragments instead of full-length MAP4; the schematic structures of these fragments are shown in Fig. 1A. The motor domain fragment of dimeric human kinesin-1 (residues 1–560) was used as the kinesin motor. All proteins were purified to homogeneity (Fig. 1B).

Effects of MAP4 Isoforms on Kinesin-driven Microtubule Gliding—Under the standard assay conditions (*10*), the gliding of microtubules could not be measured because the microtubules detached from the kinesin-coated glass surfaces following the addition of the MAP4 isoforms (data not shown). Since the presence of MAP2 or tau is reported to reduce the attachment frequency of kinesin to microtubules (*13*), the MAP4 isoforms may have shown a similar effect; this resulted in the detachment of microtubules from the glass surfaces. Although lowering the ionic strength to 0 mM KCl was partially effective in retaining the microtubules, the gliding velocity was also remarkably affected (a reduction of ~80%); this was unfavourable for

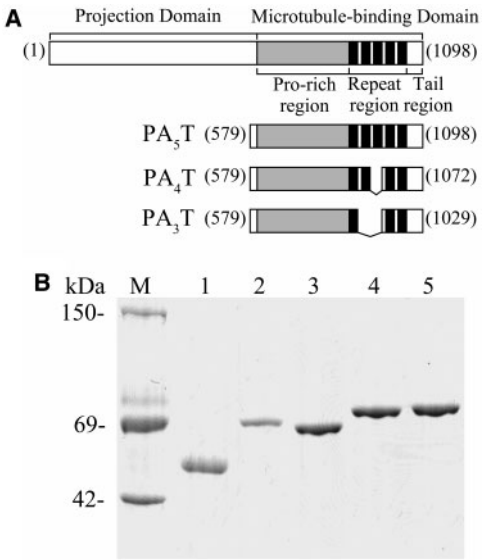

Fig. 1. **Schematic structures of the MAP4 isoform fragments, and representative SDS-PAGE patterns of the proteins used in this study.** (A) Schematic structures of intact MAP4, PA_5T (residues 579–1098), PA_4T (residues 579–1072) and PA_3T (residues 579–1029). (B) SDS-PAGE of the purified proteins. Lane M is the molecular marker: *Aspergillus niger* glucose oxidase (150 kDa), bovine serum albumin (69 kDa) and rabbit actin (42 kDa). Lane 1, tubulin; lane 2, kinesin motor domain; lane 3, PA_3T; lane 4, PA_4T; lane 5, PA_5T.

the assay. In the study of actin-myosin motors, methylcellulose is added to suppress the detachment of actin filaments from myosin-coated glass surfaces due to Brownian motion (*14*, *15*). Following the same principle, methylcellulose was added to the reaction mixtures; the detachment of microtubules from the glass surface was greatly suppressed, while the effect on the gliding velocity was lower than that in the low ionic strength conditions (a reduction of ~30%). Additionally, we considered that the viscous environment created by the presence of methylcellulose is similar to the physiological conditions because the eukaryotic cytoplasm that contains elaborate cytoskeletal structures (*1*) could also be viscous. Therefore, we adopted the methylcellulose method in the subsequent experiments.

First, we examined whether an excess amount (13.5 μM) of the MAP4 isoforms affected kinesin-driven microtubule gliding (Supplementary Movie 1a–1d). Based on the density of gliding microtubules, we estimated that the concentration of tubulin dimers that formed microtubules in this assay was ~10 nM, while the Kd values of the binding of the MAP4 isoforms to microtubules were 0.3–0.4 μM (*3*). Consequently, when the MAP4 isoforms were perfused at a concentration of 13.5 μM, it was assumed that most of the binding sites (98%) on the microtubule surface would be occupied by the MAP4 isoforms. Although the perfusion of the ATP solution alone did not affect the movement of microtubules (Supplementary Movie 1a), many microtubules decelerated or stopped when the solution was perfused by the ATP solution containing PA_5T (Supplementary Movie 1b). The decelerated microtubules repeatedly stopped and resumed their movements; their motions were not smooth. The ATP solutions containing PA_4T (Supplementary Movie 1c) or PA_3T (Supplementary Movie 1d) did not show a noticeable effect on the gliding of microtubules, although a few microtubules detached from the glass surface.

To show the effects of MAP4 isoforms more clearly, the movements of individual microtubules in the supplementary movies (Supplementary Movie 2a–d) were traced, and the distances from the original positions were plotted as a function of time (Fig. 2). In the control sample (Fig. 2A), the microtubules continuously moved at a constant rate. On the other hand, the microtubules irregularly paused and resumed (Fig. 2B; arrows) their movements in the presence of 10 μM PA_5T. Judging from the slopes of the moving phase, the microtubules moved at a constant rate in the moving phase. Although we used the expression 'decelerated' in the experiment above, the actual difference of the movement in the presence of PA_5T is the frequent appearance of the dwell of the movement. In this scenario, a few microtubules showed similar movements in the presence of PA_4T and PA_3T (Fig. 2C and D; arrows), i.e. they occasionally stopped and resumed their movements with no deceleration in the moving phase. The jerky movement of microtubules can be explained by assuming that the kinesin motors stop their motion when they encounter an MAP or a MAP-clustered region on the surface of the moving microtubules and subsequently resume their motion by an unknown mechanism.

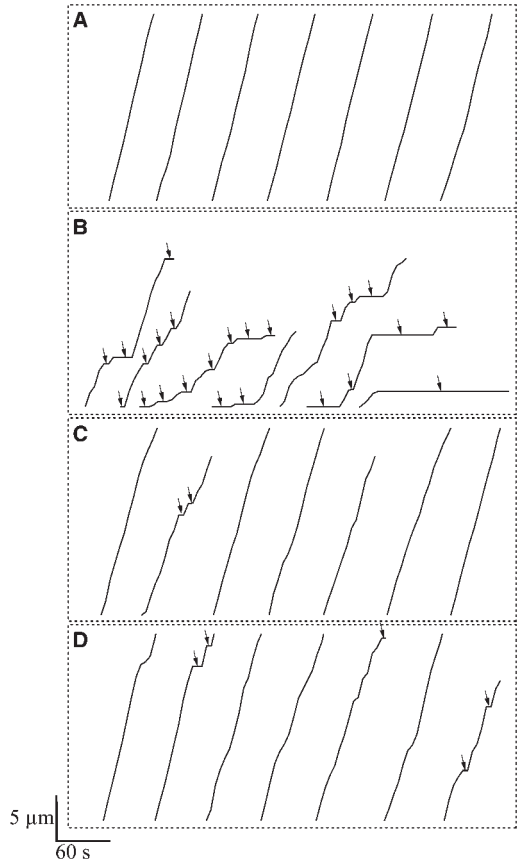

Fig. 2. **Time course of the movements of individual microtubules in the *in vitro* gliding assay.** The movements of the microtubules in the absence (A) or presence of excess amount of the MAP4 isoforms [10 μM each of PA_5T (B), PA_4T (C) and PA_3T (D)] were traced, and the distances from the original positions were plotted as a function of time. Only the representative plots are shown. Arrows indicate the position at which the smooth movement was perturbed.

Although we traced the moving microtubules in Fig. 2 to examine their movements, a considerable number of microtubules completely stopped their movements or did not move at all in the presence of the MAP isoforms, as shown in the supplementary movie. We then quantitated the percentage of the stopped microtubules that had remained immobile during a 30-s period in presence of the MAP4 isoforms. When the ATP solution containing no MAP4 isoforms was perfused, >95% microtubules were gliding smoothly (Fig. 3). Approximately 60% microtubules stopped their movements in the presence of 10 μM PA_5T (Fig. 3). Although the addition of PA_3T and PA_4T also decreased the number of moving microtubules, the effects were less prominent (Fig. 3). These results showed that the presence of the MAP4 isoforms stopped microtubule gliding in a concentration-dependent manner, with PA_5T being the most effective isoform. Similar results were observed in another independent assay using other proteins (data not shown).

Next, we analysed the moving population of microtubules by tracing them for 25 s, and measured the distances from their original positions (Fig. 4 and Table 1).

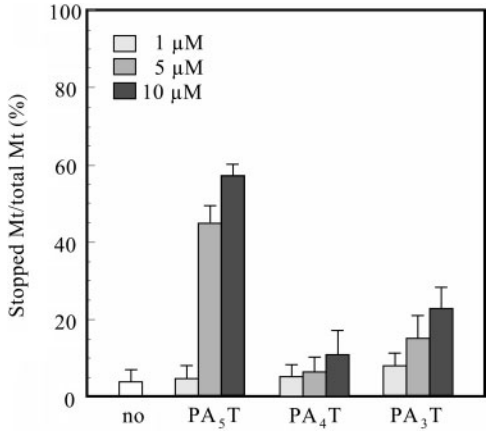

Fig. 3. **The frequency of stopped microtubules in the presence of the MAP4 isoforms.** The number of total microtubules that were initially observed in a field and stopped microtubules that remained immobile during a 30-s period were counted in randomly selected five fields of 1250 μm^2 each. Approximately 50 microtubules were observed per field. The ordinate represents the percentage of stopped microtubules in total microtubules. Error bars denote SDs.

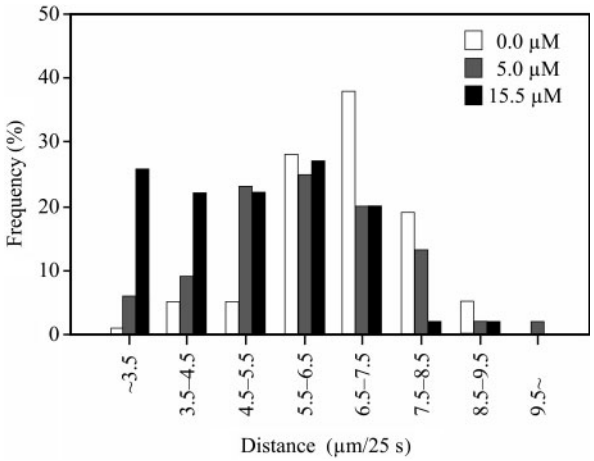

Fig. 4. **The length distributions of the tracks of moving microtubules in the presence of PA_5T.** Various concentrations (0.0, 5.0 and 15.5 μM) of PA_5T were added to the microtubule samples, and their kinesin-dependent glidings were observed. The movements of individual microtubules were tracked during a 25-s period, the distances from the original positions were measured and the frequencies were expressed as a histogram. The total number of microtubules measured was ~100 for each concentration of PA_5T. The microtubules that remained at their original positions were excluded from the data.

The mean distance in the control assay was 6.5 ± 1.1 μm (Table 1), and the figure shows a normal distribution (Fig. 4; unshaded boxes), indicating that the microtubules moved at a constant velocity of ~0.26 μm/s. When PA_5T was present, the population of microtubules that had run a shorter distance increased (Fig. 4; gray and black boxes), resulting in a shorter mean distance and a larger SD (Table 1). Since the velocity of the microtubules in the moving phase was the same as that of the control, as shown in Fig. 2, the result can be interpreted as the increase in the number of microtubules that recurrently stopped and resumed their movements in the presence of PA_5T. PA_4T and PA_3T also affected the mean distance and SDs; however, these effects were not significant (Table 1). It is evident the total dwell time in the presence of PA_5T was longer than those in the presence of PA_4T and PA_3T. The results shown in Fig. 2, Fig. 3 and Fig. 4 clearly indicated that the kinesin-driven microtubule gliding is impeded or inhibited by the presence of one of the MAP4 isoforms, i.e. PA_5T.

Table 1. **Mean distances (μm/25 s) of the moving microtubules in the presence of the MAP4 isoforms.**

MAP4 conc. [μM]	0	1	5	15.5
PA_5T	6.5 ± 1.1	6.7 ± 1.3	5.7 ± 1.6	4.7 ± 1.8
PA_4T	–	7.0 ± 1.2	6.7 ± 1.1	6.7 ± 1.3
PA_3T	–	6.7 ± 1.2	7.2 ± 1.2	7.0 ± 1.3

The Effect of MAP4 Isoforms on the Binding of Kinesin to Microtubules—Finally, we performed a sedimentation assay to determine whether the MAP4 isoforms interfere with the binding of kinesin to microtubules. As shown in Fig. 5, the binding of the MAP4 isoforms to microtubules increased with an increase in the concentrations of the added isoforms, and the excess isoforms that could not bind to microtubules were recovered in the supernatant fraction (Fig. 5; lane 4S), as expected from the stoichiometries between the isoforms and microtubules [the number of MAP4 isoforms per tubulin dimer is approximately 1 (*3*)]. In the meantime, the amount of microtubule-bound kinesin was not affected by the addition of the MAP4 isoforms (Fig. 5; lane 4S), suggesting that the MAP4 isoforms do not compete with kinesin for binding to microtubules. The competition between PA_3T and kinesin could not be judged due to the electrophoretic mobilities of the two proteins were similar (data not shown). The absence of competition between PA_5T and kinesin indicated that kinesin can bind to the PA_5T-bound microtubules although it cannot move on them.

DISCUSSION

In this study, we demonstrated that the presence of PA_5T but not PA_4T and PA_3T inhibited kinesin-driven microtubule gliding (Figs. 2 and 3). We reported elsewhere that the binding of MAP4 isoforms differentially altered the surface properties of microtubules (*3*). It is likely that the inhibition of the kinesin-driven movement was due to the PA_5T-specific modification of the microtubule surface properties. Although the Kd values of the binding of three isoforms to microtubules were different [0.42, 0.39 and 0.27 for PA_3T, PA_4T and PA_5T, respectively (*3*)], the difference in the affinities does not explain their different effects on microtubule gliding. Despite the high concentration of the MAP4 isoforms that was sufficient to cover the microtubule surfaces as mentioned earlier, PA_5T alone showed a marked effect. In addition, PA_3T was slightly more effective than PA_4T; this finding is opposite to the

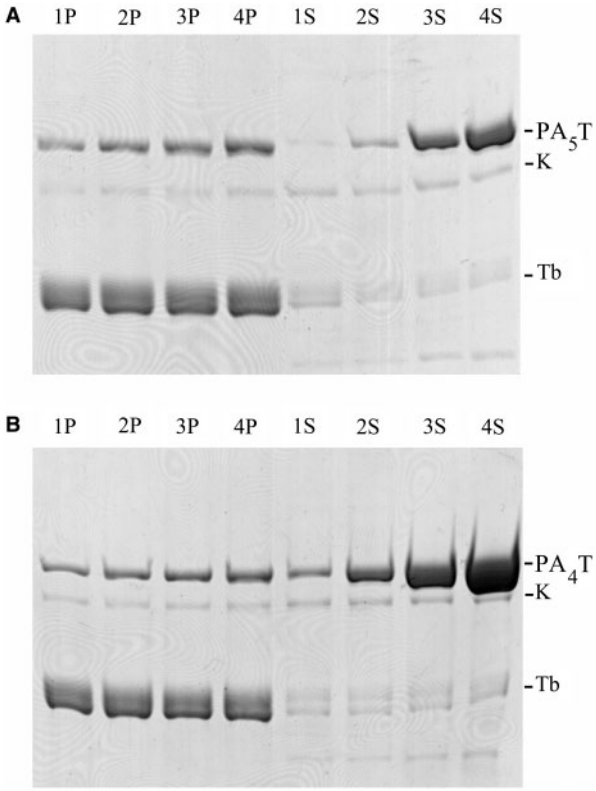

Fig. 5. **Competition between kinesin and the MAP4 isoforms for binding to microtubules.** Various concentrations (lane 1, 1 μM; lane 2, 2 μM; lane 3, 4 μM; lane 4, 8 μM) of PA_5T (A) or PA_4T (B) and a constant concentration of kinesin (2 μM) were mixed with the taxol-stabilized microtubules (5 μM). The mixtures were incubated and centrifuged. P and S are pellets and supernatants, respectively. PA_5T, PA_4T, K and Tb represent the electrophoretic positions of PA_5T, PA_4T, kinesin and tubulin, respectively.

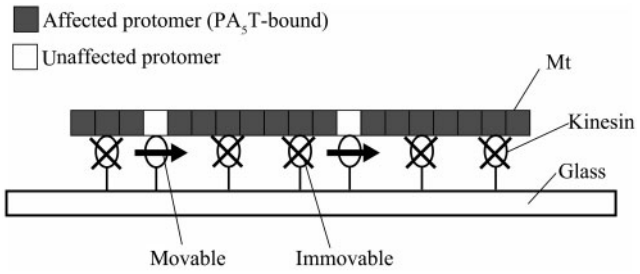

Fig. 6. **A model for the inhibition mechanism of kinesin-driven microtubule gliding by PA_5T.** PA_5T binds to microtubules; this is accompanied with changes in the microtubule surface properties (affected protomer). When an excess amount (10 μM) of PA_5T is present (Figs 2 and 3), ~2% of the tubulin protomers are estimated to be unaffected. In the conditions used in this study, many kinesin molecules simultaneously bind to a microtubule along its length, regardless of the status of protomers (affected or unaffected). A microtubule can move only when there is an interaction of some kinesins with the unaffected protomers. The active kinesin motors stop their movements when they encounter the affected protomers, although they remain bound to the microtubules. When none of the kinesins binds to the unaffected protomers in a microtubule, the microtubule cannot move. Since the binding between MAPs and microtubules is an equilibrium reaction, some MAP molecules will dissociate from the microtubule at the next moment in time. The tubulin protomers that were retained in the microtubule restore their original property (unaffected protomers), and the kinesins that remained bound to these protomers become active. Thus, the gliding of the microtubule resumes, and it will stop again when all the unaffected protomers in a microtubule lose their bound kinesin. In a living cell, the concentrations of kinesin and MAP4 are considerably lower than the present conditions. Consequently, it is possible that the kinesin-bound vesicles generally move smoothly along the microtubules but will stop when they encounter MAP4-clustered region on a microtubule.

order of their affinities. One might suspect that the PA_5T-bound microtubules attached to the glass surface more firmly than the other isoforms-bound microtubules, thereby inhibiting the movement. However, all MAP4 isoforms, including PA_5T, induced the detachment of microtubules from the glass surface when the density of kinesin was low (data not shown); this indicates that PA_5T did not augment the microtubule-glass surface interaction (data not shown). In addition, the results cannot be explained by a simple steric hindrance mechanism because the molecular sizes of the isoforms are not considerably different as expected from the differences in the inhibitory effect. Consequently, the most likely explanation for our results is that the MAP4-induced modification of the microtubule surface properties regulates the kinesin motor activity.

In the presence of PA_5T, microtubules repeatedly paused and resumed their movements (Fig. 2). The sedimentation assay (Fig. 5) revealed that kinesin can bind to microtubules covered by PA_5T. Based on these results, we propose a plausible model that explains how kinesin-driven microtubule gliding is inhibited by the presence of PA_5T (Fig. 6). A microtubule simultaneously interacts with many kinesin molecules, and it moves only when there is an interaction of some kinesins with the unaffected tubulin protomers. Although the molecular basis for the affected or unaffected state is unclear at present, one possible mechanism is the neutralization of the acidic charge of the tubulin protomer by the most basical PA_5T, as we have reported elsewhere (*3*). Electrostatic interactions between the kinesin motor domain and the microtubule surface are reportedly important for the movement (*16*, *17*).

To date, two types of regulatory mechanisms have been reported for the kinesin motors; these include regulation by cargo binding and by phosphorylation. Kinesin-I, which folds into an inactive, compact conformation with its cargo-binding tail, is reported to unfold and recover its motor activity by the binding of the cargo (*18*). The phosphorylation of conventional kinesin reportedly inhibits kinesin-vesicle binding (*19*). These regulations are, however, specific to the respective motors, and the more versatile regulators, i.e. the rail-binding regulatory modules, have not yet been reported. This is the first study to report on the regulation of a microtubule motor by a rail-binding regulatory module. It also provides an insight into the physiological functions of

the MAP4 isoforms. Although various isoforms of MAP2/MAP4/tau superfamily members have been reported (*20*), the appropriate function of each isoform remains unclear.

What types of physiological events can be driven by the inhibition of kinesin motors? In a eukaryotic cell, the kinesin motors pull the ER membranes along the microtubules up to the cell periphery (*21*, *22*). If kinesins continue to carry the ER membranes towards the periphery, most of the ER membranes will be accumulated at the cell periphery. In order to properly organize the cytoplasm, the motor proteins should not transport the vesicles continuously in one direction but should appropriately position them. Although MAP4 concentration in the living cells is lower than that in this study, MAPs inhomogeneously bind to and form clusters on microtubules (*23*, *24*). The kinesin-bound organelles would stop their movement when they encounter a cluster of PA_5T. This may be the manner in which the rail-binding regulator facilitates the appropriate organization of the cytoplasm.

MAP2, MAP4 and tau are grouped into the MAP2/MAP4/tau superfamily based on their structural similarity (*20*). Each member of the superfamily has a homologous repeat region with tandemly organized repeat sequence. The variations in the repeat region organization are generated by alternative RNA splicings (*20*), and the resultant variants differ in the contents and arrangements of the repeat sequences. It is interesting to note that MAP4 has five-, four-, and three-repeat isoforms, while mammalian MAP2 and tau have only three- and four-repeat versions. Why is the five-repeat isoform of MAP2 and tau missing? MAP2 and tau are exclusively localized in neuronal cells (*20*). In these cells, the long distance transportation by the microtubule motor plays an important role. Three- or four-repeat isoforms of tau and MAP2 did not inhibit the kinesin movement (*13*). Our results were also consistent with this finding (Figs 2 and 3). The inhibition of kinesin motor activity by the five-repeat isoform clearly perturbs the long-distance transportation; this may explain the lack of five-repeat versions in the neuron-specific MAPs, namely, MAP2 and tau.

In this study, by using a kinesin-driven microtubule gliding system, we proved our hypothesis that the MAP4-specific five-repeat isoform is a rail-binding regulator of microtubule motors. Since we used conventional kinesin in this study, the next step is to test whether the other types of kinesin motors are regulated by the same mechanism. It would be also interesting to examine the effect of MAPs on dynein—another type of microtubule motor that moves in the opposite direction. To prove the physiological roles of the MAP4-dependent regulation of the kinesin motility, future studies are required that include the real-time observations of MAP4 localization and organelle transport in a living cell.

Supplementary data are available at JB online.

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