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MCAK, a Kin I kinesin, increases the catastrophe frequency of steady-state HeLa cell microtubules in an ATP-dependent manner in vitro

Cori N. Newton[a,1], Michael Wagenbach[b,2], Yulia Ovechkina[b,2], Linda Wordeman[b,2], Leslie Wilson[a,*]

[a] *Department of Molecular, Cellular, and Developmental Biology, and The Neuroscience Research Institute, University of California, Santa Barbara, CA 93106, USA*
[b] *Department of Physiology and Biophysics, University of Washington School of Medicine, Seattle, WA USA*

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Abstract Mitotic-centromere-associated kinesin (MCAK) is a member of the KIN I (internal motor domain) subfamily of kinesin related proteins. MCAK and its homologues destabilize microtubules both in cells and in vitro. Here, we analyzed the effects of MCAK in the presence and absence of ATP on the dynamic instability behavior of steady state microtubules assembled from purified HeLa cell tubulin. In the presence of ATP, substoichiometric levels of full length MCAK and a segment (A182) consisting of the motor and neck domains strongly increased the catastrophe frequency of the microtubules. These data demonstrate that MCAK is a microtubule-catastrophe promoting factor in vitro, and support the hypothesis that MCAK may serve as a catastrophe-promoting factor in cells.

Keywords: Microtubule dynamic instability; Regulation of microtubule dynamics; Catastrophe promoting factor

1. Introduction

Dynamic instability, the switching at microtubule ends between growth and shortening [1–3], is critical for many microtubule-mediated cellular processes including chromosome movements during mitosis and the development of cell polarity [4,5]. Although dynamic instability is an intrinsic property of microtubules composed solely of tubulin, the dynamic instability behavior of microtubules in cells is considerably greater than that of microtubules composed of pure tubulin in vitro [6–10]. Specifically, microtubules assembled from purified HeLa cell tubulin in vitro display highly attenuated dynamic instability behavior, indicating that the robust dynamic instability behavior displayed by microtubules in dividing cells is created by regulatory proteins [10].

A protein that appears to destabilize microtubules and increase microtubule dynamic instability in cells is mitotic-centromere-associated kinesin (MCAK) a member of the KIN I (internal motor domain) subfamily of kinesin-related proteins [11,12]. Unlike most other kinesins that utilize ATP hydrolysis to move along microtubules, MCAK and its KIN I family members exhibit no motile activity [12–16]. Instead, they induce microtubule depolymerization in vitro in an ATP dependent manner [14,17] and in cells [16,18–20]. Specifically, incubation of MCAK with taxol- or GMPPNP stabilized microtubules in vitro induces depolymerization [16,17,19], as does the overexpression of MCAK in CHO cells [16,18]. Furthermore, immunodepletion or inactivation of XKCM1 (the Xenopus homologue of MCAK) by antibody addition in frog extracts induces the formation of abnormally long non-dynamic microtubules, supporting the idea that one of XKCM1's functions is to increase the dynamic instability of microtubules [13]. Direct analysis of the changes in the dynamics of axoneme-seeded microtubules incubated in the immunodepleted frog extracts revealed a 4-fold decrease in the catastrophe frequency (switching from growth to shortening), supporting the idea that XKCM1 might have catastrophe-promoting activity in cells [13]. In further support of this idea, overexpression of XKCM1 in PtK2 interphase cells enhanced the catastrophe frequency by 4.5-fold [20].

The effects of MCAK and its homologues on the dynamic instability of non-stabilized microtubules in vitro in the absence of other regulatory proteins at steady state have not been described. Thus, we determined the effects of MCAK on the individual dynamic parameters at plus ends of purified steady state microtubules in vitro, using highly purified HeLa cell microtubules, which exhibit intrinsically tempered dynamics. We found that substoichiometric quantities of full length MCAK potently and selectively increased the plus-end catastrophe frequency in an ATP-dependent manner, supporting the hypothesis that MCAK binds to the plus ends to convert the microtubule from a growing or attenuated (paused) state to a shortening state. In contrast to the ATP-dependent effects of MCAK on the catastrophe frequency, MCAK reduced the microtubule polymer mass in the absence of ATP. Thus, the ability of MCAK to destabilize microtubules (decrease polymer mass) and act as a catastrophe-promoting factor may be brought about by more than a single mechanism.

* Corresponding author. Fax: +805-893-8094.
E-mail address: wilson@lifesci.ucsb.edu (L. Wilson).

[1] Fax: +805-893-8094.
[2] Fax: +206-685-0619.

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2. Materials and methods

2.1. MCAK expression, HeLa cell tubulin purification, determination of MCAK concentrations

Full-length hamster MCAK (FL-MCAK) was expressed in baculovirus and purified as previously described [18]. The hamster MCAK motor domain and the A182 fragment were bacterially expressed and purified as described in [19]. Purified HeLa cell tubulin was isolated as described by Newton et al. [10]. HeLa cell tubulin and the MCAK proteins were exchanged into 80 mM Pipes, 1 mM $MgCl_2$, 1 mM EGTA, 0.5% NP-40, and 60 mM KCl, pH 6.8 [BRB80], by concentrating the solutions in a Biomax-30 centrifugal filter (Millipore) and by centrifugation in a Sorvall centrifuge (SS34 rotor, 10 000 rpm, 4 °C). Aliquots of the protein solutions were frozen in liquid nitrogen and stored at −70 °C until use. The concentrations of active FL-MCAK dimer, A182, and the motor domain negative control were determined by measuring their ATP binding activities [21]. The ratios of active (able to bind ATP) to inactive (unable to bind ATP) for FL-MCAK, A182, and the motor domain were 1:5. FL-MCAK activity is reported as a dimer (two ATP binding sites per dimer), while the protein concentrations of A182 and the core motor domain proteins are reported as the monomers [21]. MCAK inhibits microtubule polymerization and thus two criteria were used to determine the concentrations of the MCAK proteins used for the dynamics experiments; the highest concentration at which we obtained adequate numbers and lengths of assembled microtubules, and the concentration at which we could detect a change in dynamic instability behavior as compared with the inactive motor domain control. Tubulin concentration was determined by the Bradford method using bovine serum albumin as the standard [22].

2.2. Determination of HeLa cell microtubule dynamic instability in the presence of full-length MCAK, A182, and the MCAK Motor Domain

Samples containing 15.5 μM tubulin and 58.9 nM FL-MCAK, 3.5 nM A182 or 30 nM core MCAK motor domain (see Fig. 1) were mixed with 0.5 mg/ml casein in BRB80 buffer containing 1 mM GTP and 1 mM DTT and incubated on ice for no more than 15 min. ATP, when used, was added at a final concentration of 2 mM. Flow-through chambers were assembled with double-stick tape and pre-cleaned glass coverslips and slides. Diluted *Strongylocentrotus purpuratus* axonemes were flowed through the chambers and after incubation on ice for 5 min, unattached axonemes were washed out by addition of 5 mg/ml casein in BRB80 buffer and slides were further incubated to allow binding of casein to the chamber surfaces. The chambers were then washed with BRB80 buffer. The pre-mixed sample containing HeLa cell tubulin and MCAK was then flowed through the chamber and sealed with 1:1:1 vasoline, lanolin, and paraffin. The slide was placed on a pre-warmed (35 °C) differential interference contrast (DIC) microscope stage and incubated for 30 min to reach steady-state. Individual microtubules were then imaged and analyzed at 35 °C as previously described [10].

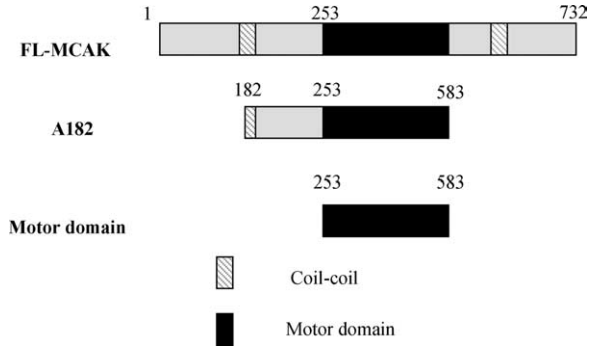

Fig. 1. Schematic showing the different MCAK constructs used. FL is full-length Hamster MCAK (732 amino acids). A182 is a truncated form of MCAK which contains the motor domain and the region amino-terminal to the motor domain (amino acids 182–583). The motor domain is the core motor domain of MCAK only (amino acids 253–583).

3. Results

3.1. Effects of MCAK on the Dynamic instability of HeLa cell microtubules

In an effort to understand MCAK's role as a regulator of microtubule dynamics, we analyzed the effects of three MCAK constructs in the presence and absence of 2 mM ATP on the dynamic instability behavior of steady state microtubules composed of HeLa cell tubulin (15.5 μM). These microtubules display intrinsically tempered dynamic instability behavior [10], making them very useful for analysis of regulators that increase dynamics. The concentration of active FL MCAK dimer (Fig. 1) chosen for use in this study was 59 nM. This concentration was the highest concentration that yielded sufficient polymer mass and microtubule lengths for analysis. We also analyzed the effects of a fragment called A182 at 3.5 nM using the same criteria for choice of concentration (containing the core motor domain between residues 253 and 583 and a region near the amino-terminus from residue 182 to 253 called the "neck domain") and, as a negative control, a fragment consisting only of the core motor domain (30 nM) which is devoid of microtubule depolymerizing activity [16,19] (Fig. 1).

Life-history traces showing length changes with time of microtubules assembled in the presence of 2 mM ATP with the inactive core motor domain, FL-MCAK, and A182, are shown in Fig. 2. The traces demonstrate that microtubules assembled with the inactive core motor domain (Fig. 2A) display minimal dynamic instability behavior, as do control HeLa cell microtubules composed solely of tubulin (data not shown) [10]. In contrast with microtubules assembled from brain tubulin, microtubules assembled from highly purified HeLa cell tubulin rarely display growth or shortening or transitions to shortening (catastrophes) [10]. By comparison, both FL-MCAK (Fig. 2B) and the active A182 fragment (Fig. 2C) significantly increased the catastrophe frequency.

The effects of the three MCAK constructs on the individual steady state dynamic instability parameters in the presence and absence of 2 mM ATP are shown quantitatively in Table 1. Neither FL-MCAK nor A182 significantly changed the growth or shortening rates as compared with the inactive motor domain, nor did they induce significant differences in the mean length grown per growth event or the mean length shortened per shortening event (99% confidence level, Student's *t* test). However, both FL-MCAK and the active A182 fragment significantly increased the catastrophe frequency. Specifically, low ratios both of dimeric FL-MCAK and monomeric A182 (1/263 moles of active FL-MCAK/mole of tubulin and 1:4428 moles of A182/mole of tubulin) increased the catastrophe frequency by ~7-fold (from 0.03 min^{-1} with the inactive motor domain to 0.2 min^{-1}, both with FL-MCAK and A182, Table 1). Interestingly, the monomeric A182 exerted much more potent catastrophe promoting activity than the dimeric FL-MCAK, as 16.8-fold more FL-MCAK exerted the same catastrophe-promoting activity as A182. In addition, it appears that both FL-MCAK and A182 decreased the rescue frequency (frequency of transitions from shortening to either growth or an attenuated state) by 61% and 46%, respectively (Table 1). As a result of the increased catastrophe frequency, microtubules in the presence of FL-MCAK and A182 spent ~88% more time shortening than in the presence of the motor domain control (Table 1). In addition, as would be expected with a factor that increases the catastrophe frequency thereby

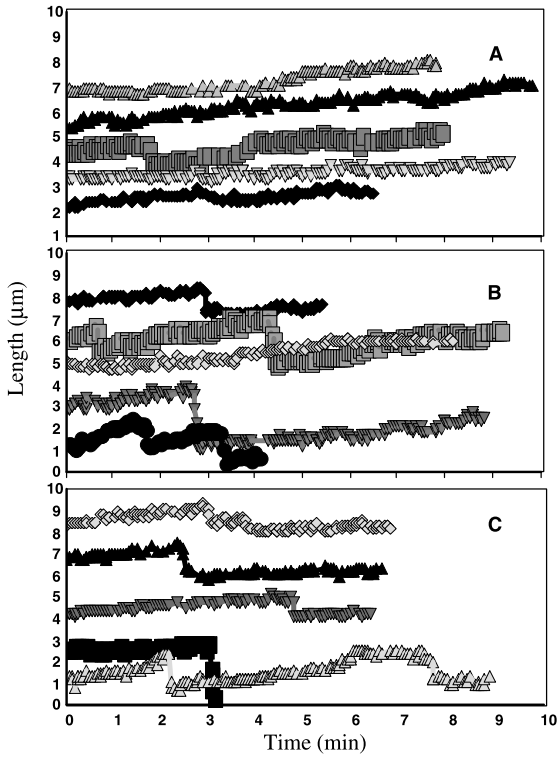

Fig. 2. Life-history traces of individual axoneme-seeded HeLa cell microtubules. Panel A, microtubules incubated with the core motor domain (30 nM). Panel B, microtubules incubated with FL-MCAK (58.9 nM). Panel C, microtubules in the presence of A182 (3.5 nM). The tubulin concentration was 15.5 μM.

increasing the rate tubulin would appear in the soluble pool, MCAK and A182 increased the percentage of time the microtubules grew (by 33% and 21%, respectively) (Table 1). Finally, FL-MCAK and A182 increased the dynamicity (overall detectable growth and shortening per unit time) by ~2.5-fold as compared to the motor domain control (Table 1).

3.2. The catastrophe promoting activity of MCAK and A182 requires ATP

The catastrophe-promoting activity of FL-MCAK and A182 MCAK requires ATP (Table 1), confirming suggestions of previous studies [14,17,23]. In the absence of any added ATP, the catastrophe frequency with FL- MCAK or A182 was no different than the catastrophe frequency with the core motor domain (Table 1). These experiments also show that GTP, which was present at a concentration of 1 mM, could not substitute for ATP.

3.3. Microtubule length changes in the presence of MCAK

In preliminary experiments we found that MCAK and A182, but not the motor domain which had similar lengths and numbers of microtubules as buffer alone (unpublished data),

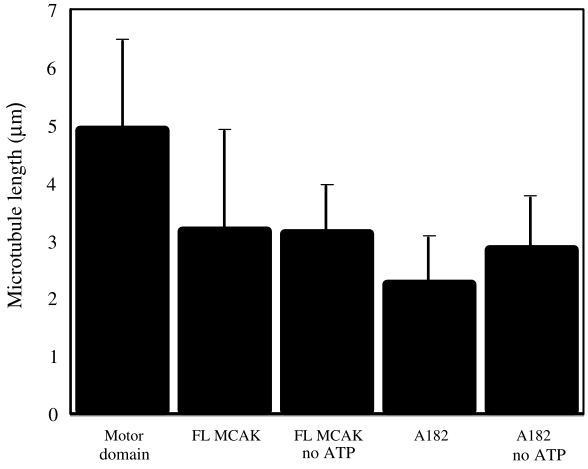

Fig. 3. Average microtubule length for axoneme-seeded HeLa cell microtubules. The microtubules were measured in the following conditions (shown left to right), in the presence of the core motor domain (1:520 mole of motor domain: mole of tubulin), in the presence of FL-MCAK (1:260 mole of FL-MCAK: mole of tubulin) ± ATP, and in the presence of A182 (1:4400 mole of A182: mole of tubulin) ± ATP.

Table 1
Dynamic instability parameters of HeLa cell microtubules in the presence of MCAK's core motor domain, A182 and FL-MCAK

Parameter	Motor domain (plus ATP)	FL (plus ATP)	FL (no ATP)	A182 (plus ATP)	A182 (no ATP)
Rate (μm/min)					
Growing	0.39 ± 0.4	0.38 ± 0.2	0.36 ± 0.3	0.42 ± 0.3	0.32 ± 0.2
Shortening	23.4 ± 9.8	16.5 ± 9.4	18.3 ± 6.3	13.4 ± 7.8	13.9 ± 11.6
Mean length (μm/event)					
Growing	0.6 ± 0.4	0.7 ± 0.4	0.6 ± 0.5	0.5 ± 0.3	0.5 ± 0.3
Shortening	1.1 ± 0.9	1.5 ± 1.3	1.1 ± 0.4	0.9 ± 0.6	0.9 ± 0.4
Transition frequencies (min^{-1})					
Catastrophe	0.03	0.2	0.04	0.2	0.03
Rescue	20.0	7.8	15.4	10.7	11.1
Percentage of time					
Growing	33.0	49.1	26.2	41.3	30.7
Shortening	0.2	1.6	0.2	1.8	0.3
Attenuated	66.8	49.3	73.6	56.9	69.0
Dynamicity (μm/min)	0.17	0.45	0.14	0.41	0.14

Changes in length at plus ends of microtubules assembled with 15.5 μM HeLa cell tubulin in the presence of 30 nM MCAK core motor domain, 58.9 nM FL-MCAK and 3.5 nM A182 were measured for a total of 205, 160 and 153 min, respectively. Values are shown as means ± S.D. The rates and mean lengths were not significantly different (99% confidence level, Student's *t* test).

reduced the lengths and numbers of microtubules assembled at the ends of axonemal seeds. Thus, to measure the effects of MCAK and A182 on the dynamic instability of microtubules, we chose to use the highest concentration of FL-MCAK and A182 that still yielded adequate numbers and lengths of microtubules at the ends of the seeds (59 nM FL-MCAK and 3.5 nM A182). At the concentrations used, MCAK and A182 reduced the lengths of the microtubules in the presence of ATP. Surprisingly, the microtubule lengths were also reduced in the absence of ATP (Fig. 3). For example, MCAK reduced the mean length to a similar extent, by 37%, either in the presence or absence of ATP. MCAK and A182 also reduced the number of microtubules polymerized at the ends of the axoneme-seeds either in the presence or absence of ATP as compared with the number of microtubules assembled in the presence of the motor domain control (data not shown).

4. Discussion

Previous work from a number of laboratories has shown that MCAK and other members of the Kin I family of internal motor domain kinesins, including XKCM1 and the neuronal family member XKIF2, do not "walk" along microtubules as do most other kinesins, but rather, destabilize microtubules apparently by acting at the microtubule ends [14,16–20]. MCAK and its homologues bind to microtubules preferentially at their ends [14,17,23] and their localization at the kinetochores of mitotic chromosomes and at spindle poles during mitosis places them near the ends [11,18,20]. Considerable evidence indicates that MCAK induces microtubule depolymerization both in cells [16,18] and in vitro [16,17,19]. In addition, evidence both in cells and with microtubules reconstituted in vitro has suggested that MCAK homologues can act as catastrophe-promoting factors [15,20]. For example, overexpression of XKCM1 in PtK2 cells increases the catastrophe frequency of microtubules [20] and depletion or inhibition of XKCM1 in *Xenopus* egg extracts induces formation of large asters with abnormally long, non-dynamic microtubules [13]. The strongest direct evidence indicating that an MCAK homologue is a bona fide catastrophe factor was obtained under non-steady state conditions [15]. However, in order to demonstrate unambiguously that MCAK is a bone fide catastrophe factor, it is necessary to measure the catastrophe promoting activity under conditions in which the microtubules are at or near steady state, with the soluble tubulin concentration constant. For example, under non-steady state conditions, a tubulin-sequestering factor would decrease the soluble tubulin concentration, which would result in net microtubule disassembly and induce a transient increase in the catastrophe frequency.

In the present study, we analyzed the effects of MCAK and A182, consisting of the motor and neck domains, on the individual dynamic instability parameters of microtubules assembled to steady state in the presence of the Kin I proteins. We used microtubules that were assembled from highly purified tubulin, from dividing cells, and thus a normal substrate for MCAK. Finally, the microtubules were analyzed in the absence of any other regulatory proteins, so the effects of MCAK alone could be determined with microtubules that in the absence of MCAK display highly tempered dynamic instability behavior. We find that MCAK acts as a potent microtubule catastrophe-factor in vitro, increasing the switching frequency from the growing or attenuated (paused) state without exerting significant effects on the rate or extent of steady state growth or shortening.

4.1. Catastrophe promoting activity of MCAK and A182 at steady state in vitro

In the presence of inactive MCAK motor domain, the purified HeLa cell microtubules rarely switched from the growing or an attenuated state to shortening (Table 1, 0.03 per min, or only once every 33 min). Essentially, the same catastrophe frequency was obtained with steady state HeLa cell microtubules in the absence of any regulatory proteins (1 every 50 min) [10]. In contrast, only 1 molecule of FL-MCAK dimer per 263 molecules of tubulin increased the catastrophe frequency by $\sim$6.7-fold to $\sim$ once every 5 min. The MCAK-induced increase in the catastrophe frequency was not high enough to cause an increase in the soluble tubulin level and thus increase the growth rate. These results further support the conclusion that the microtubules were at a true steady state. The observed reduction in the rescue frequency in the presence of MCAK is probably not meaningful because the time the microtubules spent shortening was exceedingly small and the lengths of the microtubules were short, making it difficult to accurately measure transitions from shortening to growth. In accord with the observation that FL-MCAK increased the catastrophe frequency, it also increased total visibly detectable tubulin subunit exchange (dynamicity), which resulted from an increase in the percentage of time the microtubules spent shortening and growing (Table 1). Thus, it is clear that MCAK potently increases the catastrophe frequency of HeLa cell microtubules in vitro.

It is interesting to note that the catastrophe frequency of HeLa cell microtubules in the presence of MCAK is still quite low compared to the catastrophe frequency of microtubules in living cells [5,9]. While we do not know the concentration of active MCAK at microtubule ends in cells, the data support the idea that multiple regulatory factors must be necessary to achieve not only the high catastrophe frequency of cellular microtubules but also the fast rates of growth [5–10]. Additional studies involving the combination of multiple microtubule regulatory factors and purified HeLa cell microtubules would help provide further insight into the regulation of microtubule dynamics in cells.

4.2. A182 is a more potent catastrophe-promoting factor than FL-MCAK

Like full-length dimeric MCAK, the monomeric A182 increased the catastrophe frequency of the microtubules without significantly affecting the growth or shortening rates (Table 1). Surprisingly, A182 was much more potent than full-length MCAK (Table 1). Specifically, more than 16 times the amount of full-length MCAK than A182 was needed to elicit a similar increase in the catastrophe frequency. One possible explanation for the increased potency of A182 is that it is a monomer rather than a dimer, and that the monomer is more potent than the dimer. Another possibility is that the missing regions of A182 play a regulatory role in MCAK's catastrophe-promoting activity. A recent study by the Wordeman lab has shown that the C-terminal end of FL-MCAK inhibits lattice-stimulated ATPase activity of the motor supporting the idea that protein regions outside the truncated MCAK (A182) play a

regulatory role (unpublished data, Ayana Moore and Linda Wordeman). Future studies involving a detailed analysis of the relationship between MCAK structure and its catastrophe-promoting activity should help resolve this issue.

4.3. Reduction in the microtubule polymer mass by MCAK

Consistent with previous studies [14,20], MCAK reduced the length of the axoneme-seeded steady state microtubules by 36% and A182 reduced the length by 64% (Fig. 3). In addition to the reduction in the length of the microtubules, there was also a reduction in the number of microtubules assembled from the seeds. Surprisingly, the reduction in the mean lengths and microtubule number occurred both in the presence and absence of ATP, while the catastrophe-promoting activity only occurred in the presence of ATP. One possible reason for the reduction in microtubule mass could be that MCAK was binding to soluble tubulin inactivating or sequestering it. In support of this idea, several studies have shown that MCAK is capable of binding to tubulin dimers. For example, Desai et al. [14] showed that MCAK homologues could bind tubulin dimers in the presence of AMPPNP. In addition, soluble tubulin is capable of stimulating the ATPase activity of MCAK [17,23] further supporting the idea that MCAK is capable of binding to tubulin dimers. There are several plausible reasons for why MCAK might bind to soluble tubulin in our dynamics assays. One reason is that the binding of MCAK to tubulin dimer in the absence of nucleotide is a step in MCAK's enzymatic pathway. Moores et al. [23] proposed that MCAK homologues in a nucleotide free (inactive) state allow MCAK to search for a "productive binding site" such as the terminal tubulin dimers of capped microtubules. It is thought based on this model that soluble GTP-tubulin would have similar structural attributes to terminal tubulin dimers at the microtubule ends. Finally, we cannot rule out the possibility that the enzymatically inactive fraction of the MCAK in this work (unable to bind ATP) sequesters tubulin in a non-physiological manner.

4.4. How might MCAK increase the catastrophe frequency?

We found that the catastrophe-promoting activity of FL-MCAK and A182 was dependent on the presence of ATP (Table 1). To test whether ATP hydrolysis was necessary for MCAK's catastrophe promoting activity, we also analyzed the effects of MCAK on the catastrophe promoting activity in the presence of the non-hydrolyzable ATP analogue, AMPPNP (2 mM). The catastrophe-promoting activity of MCAK in the presence of AMPPNP was the same as in the absence of ATP, suggesting that ATP hydrolysis is necessary for MCAK's microtubule catastrophe-promoting activity (data not shown). These results are consistent with those of Hunter et al. [17] who found that ATP hydrolysis is necessary for MCAK's depolymerizing activity, and are consistent with the hypothesis of Moores et al. [23] that the hydrolysis of ATP is required for converting a growing microtubule end to a shortening end.

Several studies have led to the idea that MCAK and its homologues destabilize microtubules by inducing a conformational change at the end of a microtubule, which promotes microtubule shortening [14,23]. Our results also support the idea that MCAK destabilizes microtubules by acting at microtubule ends. Perhaps, MCAK specifically binds to the stabilizing tubulin-GDP-Pi or GTP cap, converting it to the strained rapidly disassembling tubulin-GDP form [24]. The observed increase in the catastrophe frequency of HeLa cell microtubules in the presence of MCAK must be due to the binding of MCAK to the microtubules. Furthermore, extremely small quantities of MCAK were capable of increasing the catastrophe frequency, suggesting that MCAK's destabilizing activity is the result of MCAK binding at microtubule ends. Quantitatively, based on our data and experimental conditions, only 1 molecule of FL-MCAK was present for every 263 tubulin dimers (both soluble tubulin and in microtubules), suggesting that for a microtubule that is 3 μm in length (1690 dimers/μm) there would be ~9 molecules available per microtubule end, indicating that only a few molecules of MCAK are needed to elicit catastrophes at the ends of microtubules. In summary, the data indicate that MCAK is a potent ATP-dependent catastrophe factor, which mechanistically binds to and destabilizes microtubule ends in vitro, supporting the idea that MCAK is a catastrophe-promoting factor in cells.

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