GTP Is Required for the Microtubule Catastrophe-Inducing Activity of MAP200, a Tobacco Homolog of XMAP215\textsuperscript{1W}

Takahiro Hamada*, Tomohiko J. Itoh, Takashi Hashimoto, Teruo Shimmen, and Seiji Sonobe

Department of Life Science, Graduate School of Life Science, University of Hyogo, Harima Science Park City, Hyogo 678–1297, Japan (T. Hamada, T.S., S.S.); Division of Biological Sciences, Graduate School of Science, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464–8602, Japan (T.J.I.); and Graduate School of Biological Sciences, Nara Institute of Science and Technology, Ikoma 630–0101, Japan (T. Hamada, T. Hashimoto)

Widely conserved among eukaryotes, the microtubule-associated protein 215 (MAP215) family enhances microtubule dynamic instability. The family member studied most extensively, Xenopus laevis XMAP215, has been reported to enhance both assembly and disassembly parameters, although the mechanism whereby one protein can exert these apparently contradictory effects has not been clarified. Here, we analyze the activity of a plant MAP215 homolog, tobacco (Nicotiana tabacum) MAP200 on microtubule behavior in vitro. We show that, like XMAP215, MAP200 promotes both assembly and disassembly parameters, including microtubule growth rate and catastrophe frequency. When MAP200 is added to tubulin and taxol, strikingly long-coiled structures form. When GDP partially replaces GTP, the increase of catastrophe frequency by MAP200 is strongly diminished, even though this replacement stimulates catastrophe in the absence of MAP200. This implies that MAP200 induces catastrophes by a specific, GTP-requiring pathway. We hypothesize that, in the presence of MAP200, a catastrophe-prone microtubule lattice forms occasionally when elongated but nonadjacent protofilaments make lateral contacts.

Microtubules switch stochastically between growth and shortening phases, a phenomenon known as dynamic instability (Mitchison and Kirschner, 1984). Switching from a growth phase to a shortening phase is an event termed catastrophe, and, conversely, switching from shortening to growth is termed rescue. Dynamic instability is essential for the function and organization of microtubule structures, allowing microtubule arrays to explore their environment and to be remodeled rapidly. Although dynamic instability can be observed in polymers created from pure tubulin, the characteristics of the phenomenon are subject to profound regulation by microtubule-associated proteins (Howard and Hyman, 2007).

In the context of regulating dynamic instability, among myriad proteins, one family, microtubule-associated protein 215 (MAP215), has been studied particularly widely (Gard et al., 2004). This family has been reported to play a major role organizing microtubule structures in many species, including: Schizosaccharomyces pombe (Ohkura et al., 1988; Garcia et al., 2001), budding yeast (Saccharomyces cerevisiae; Severin et al., 2001), Caenorhabditis elegans (Matthews et al., 1998), Xenopus laevis (Tournebize et al., 2000), Homo sapiens (Gergely et al., 2003; Cassimeris and Morabito, 2004), Drosophila melanogaster (Goshima et al., 2005), Dictyostelium discoideum (Hestermann and Graf, 2004), Aspergillus nidulans (Enke et al., 2007), and Arabidopsis (Arabidopsis thaliana; Whittington et al., 2001; Kawamura and Wasteneys, 2008). In all of these organisms, the loss-of-function phenotype can be summarized as decreased microtubule length, indicating that MAP215, as a net result, promotes microtubule assembly.

Further insight into the function of MAP215 has been gained from in vitro analysis. The extent of microtubule assembly and the rate of growth are substantially increased by Xenopus XMAP215 (Gard and Kirschner, 1987) as well as by the human ortholog, TOGp (Charrasse et al., 1998). However, interestingly, analyzing parameters of dynamic instability has revealed that XMAP215 not only promotes growth rate but also promotes shortening rate and catastrophe frequency (Vasquez et al., 1994). Catastrophe-inducing...

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* Corresponding author; e-mail hama@bs.naist.jp.

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activity was also demonstrated by finding that XMAP215 can disassemble GMPCPP-stabilized microtubules (Shirasu-Hiza et al., 2003). Consistent with the idea that this protein can enhance shortening, GFP-XMAP215 labeled both growing and shortening microtubule ends (Brouhard et al., 2008), and the budding yeast ortholog, stu2, analyzed in vitro, promotes catastrophe frequency as its major activity (van Breugel et al., 2003). The ability of a protein to enhance microtubule growth as well as to increase the frequency of catastrophe is paradoxical, and the mechanism for MAP215’s bipolar activity remains to be demonstrated.

In plants, orthologs of MAP215, identified as MICROTEBULE ORGANIZATION1 (MOR1) in Arabidopsis (Whittington et al., 2001) and as MAP200 in tobacco (Nicotiana tabacum; Yasuhara et al., 2002) are about 70% similar to their animal counterparts, indicating strong conservation. The effects of plant MAP201 on dynamic instability have not been characterized in vitro, although a net promotion of microtubule assembly has been observed for MAP200 (Hamada et al., 2004). In living cells, analysis of dynamic instability in wild-type and mor1-1 epidermal cells revealed mor1-1 mutation increases pause duration (Kawamura and Wasteneys, 2008). However, with living cells it is difficult to distinguish direct effects of the protein from indirect effects caused by the plant.

Here, we characterize dynamic instability in vitro as affected by MAP200. We confirm that the plant ortholog promotes growth, catastrophes, and rescues; however, we show that, when GDP partially replaces GTP, catastrophe promotion by MAP200 is suppressed more strongly than is growth. This result suggests that MAP215 induces catastrophe by a specific, GTP-dependent mechanism. We propose a model that predicts catastrophes promoted by MAP215 are mechanistically distinct from those arising from the loss of the GTP cap.

**RESULTS**

**MAP200 Affects Dynamic Instability at Both Microtubule Ends**

We purified endogenous MAP200 from tobacco BY-2 cells (Hamada et al., 2004; Supplemental Fig. S1) and analyzed its effect on microtubule dynamic instability (Table I; Supplemental Fig. S2; Supplemental Movie S1). At the plus end, MAP200 increased growth rate, apparently saturating at 2 μM with a promotion of around 30% relative to the control (Table I). MAP200 also increased both the catastrophe frequency and the rescue frequency by up to 50% relative to the control. Although shortening rate tended to increase to a similar extent, the difference was not significant. At the minus end, MAP200 promoted growth rate nearly twice as effectively as at the plus end but had no discernable effect on catastrophe frequency. These results show that MAP200 resembles X. laevis MAP215 in promoting growth rate and catastrophe frequency (at the plus end) but differs in having no pronounced effect on shortening rate and actually increasing rescue frequency in contrast to the decrease caused by XMAP215 (Vasquez et al., 1994).

**MAP200 Counts the Microtubule-Depolymerizing Effect of GDP**

To analyze further the effect of MAP200 on microtubule dynamics, we perturbed dynamic instability with GDP, which has been shown to inhibit tubulin polymerization proportionally to the ratio of GDP to GTP (Jameson and Caplow, 1980). We first used turbidity to examine the extent of microtubule assembly as a function of GDP-to-GTP ratio, keeping the total concentration of guanine nucleotide constant (Fig. 1). Consistent with previous work (Jameson and Caplow, 1980), as the ratio of GDP to GTP increased, microtubule assembly decreased, with roughly sigmoidal

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**Table I. Effect of MAP200 on dynamic instability**

Data are mean ± s.d. Asterisks show the significance at which the equivalence of treatment and control means are rejected: *P < 0.05, **P < 0.005. Although shortening rate at the plus end apparently increased by 40%, the difference was not significant due to high sample variance. At the minus end, shortening rate and rescue frequency were not able to be determined because the shortening microtubules were rescued within 5 s in every case. N.D., Not determined; N.S., Not significant.

<table>
<thead>
<tr>
<th>MAP200 Concentration</th>
<th>0 μM (n = 23, 97.8 min)</th>
<th>1 μM (n = 28, 110.6 min)</th>
<th>2 μM (n = 19, 90.7 min)</th>
<th>4 μM (n = 21, 70.6 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plus end</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Growth rate</td>
<td>μm/min</td>
<td>1.10 ± 0.31</td>
<td>1.26 ± 0.25 N.S.</td>
<td>1.43 ± 0.32**</td>
</tr>
<tr>
<td>Shorting rate</td>
<td>μm/min</td>
<td>25.17 ± 11.8</td>
<td>27.83 ± 7.7 N.S.</td>
<td>30.46 ± 15.5 N.S.</td>
</tr>
<tr>
<td>Catastrophe frequency</td>
<td>N/min</td>
<td>0.160 ± 0.004</td>
<td>0.165 ± 0.004 N.S.</td>
<td>0.198 ± 0.005**</td>
</tr>
<tr>
<td>Rescue frequency</td>
<td>N/min</td>
<td>1.40 ± 0.10</td>
<td>1.59 ± 0.11 N.S.</td>
<td>1.79 ± 0.17 N.S.</td>
</tr>
<tr>
<td><strong>Minus end</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Growth rate</td>
<td>μm/min</td>
<td>0.28 ± 0.23</td>
<td>0.31 ± 0.32 N.S.</td>
<td>0.46 ± 0.23*</td>
</tr>
<tr>
<td>Shorting rate</td>
<td>μm/min</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Catastrophe frequency</td>
<td>N/min</td>
<td>0.07 ± 0.009</td>
<td>0.08 ± 0.004 N.S.</td>
<td>0.07 ± 0.011 N.S.</td>
</tr>
<tr>
<td>Rescue frequency</td>
<td>N/min</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
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</table>
dependence on the ratio (Fig. 1C). In the absence of GDP, MAP200 increased microtubule assembly, consistent with previous reports (Hamada et al., 2004) and with the dynamic instability parameters shown here (Table I). Interestingly, MAP200 countered the depolymerizing effect of GDP. In the presence of MAP200, up to 25% GDP caused no decrease whatsoever in the extent of assembly and there was appreciable assembly at 75% GDP, a level at which there was essentially no assembly for controls (Fig. 1). In the presence of 100% GDP (1 mM), microtubules were not observed in the presence of MAP200, indicating that MAP200 cannot override the requirement of GTP for tubulin polymerization.

MAP200 Suppresses GDP-Induced Increase in Catastrophe Frequency

To understand further how MAP200 influences microtubule behavior, we examined the effect of MAP200 on dynamic instability in the presence of GDP (Table II; Fig. 2; Supplemental Movies S2–4; Supplemental Fig. S3). In controls, GDP decreased growth rate and rescues, and increased catastrophes, as expected and consistent with the decreased assembly measured by turbidity (Fig. 1). MAP200 enhanced the suppression of rescue caused by GDP and lessened the effect of GDP on growth rate (Table II; Fig. 2). Surprisingly, although both MAP200 and GDP promoted catastrophes on their own, MAP200 suppressed the increase in catastrophes caused by increasing ratios of GDP to GTP. At 25% GDP, catastrophe frequency was increased by about 170% for control microtubules but only by about 110% for those in the presence of MAP200. At 50% GDP, catastrophes were approximately 280% higher in controls but merely 150% higher for microtubules with MAP200. This can account for the ability of MAP200 to maintain polymer levels despite the admixture of GDP (Fig. 1) and suggests that MAP200 promotes catastrophes by a different mechanism than does GDP.

MAP200 Forms a Complex with GDP-Tubulin

To investigate further the interaction of tobacco MAP200 with microtubules, we investigated complex formation. MAP200 is known to form a complex with GTP-tubulin dimers in a sodium chloride solution (Hamada et al., 2004); here, we report that MAP200 also forms a complex with GDP-tubulin dimers in PME buffer (Fig. 3). Purified GDP-tubulin dimers were observed mainly as particles, with a few small aggregates, presumably representing tubulin oligomers (Fig. 3A). With the addition of MAP200, the number and size of the aggregates increased (Fig. 3B). Formation of a complex between MAP200 and GDP-tubulin was confirmed by Suc density gradient centrifugation, where the complex ran in high-Mr fractions with roughly one MAP200 to four tubulin dimers (Fig. 3C). Evidently, bound GTP is not required for forming a MAP200-tubulin complex.

Recently, XMAP215 was reported to form a spherical structure with one GTP tubulin dimer (Brouhard et al., 2008). We observed small, spherical structures in the mixture of MAP200 and GDP-tubulin (Fig. 3B), but we did not characterize them and similar structures were observed occasionally in the pure tubulin solution (control). Previously, it was reported that the complex

![Figure 1](https://example.com/figure1.png)
formed from MAP200 and GTP-tubulin sediments at 390,000g (Hamada et al., 2004). However, such centrifugation did not pellet the MAP200-GDP-tubulin complex (data not shown). We suspect that MAP200-GTP-tubulin complexes form aggregates, because GTP-tubulin dimers can polymerize to a small extent even in the presence of 120 mM NaCl, whereas no such polymerization is possible with GDP-tubulin subunits.

Coiled Structures Form in the Presence of MAP200, Tubulin, and Taxol

In purifying MAP200, we consistently checked microtubule bundling activity of each column fraction, by using both dark-field and electron microscopy. Fractions with bundling activity were discarded to avoid contamination from other proteins. While making these observations, we noticed curious, coiled structures (Fig. 4A). Their formation required MAP200, tubulin, and taxol. We suggest that the structures represent a pair of protofilaments because the structure is about that size, taxol is known to stabilize lateral interactions between protofilaments (Amos, 2004), and because, in favorable views, the coil has a longitudinal striation discernable along its center (Fig. 4B, arrows).

To ascertain the origin of the coiled structures, we assayed the extent of their formation when MAP200, tubulin, and taxol were combined in different order

Table II. Effect of MAP200 on dynamic instability at the microtubule plus end in the presence of various GDP-to-GTP ratios

Data report mean ± so. Asterisks show the significance at which the equivalence of treatment (+MAP200) and control (−MAP200) means are rejected: *P < 0.05, **P < 0.005. MAP200 samples contain 2 μM MAP200 at the final concentration. The differences on shorting rate were not significant due to high sample variance. Note that the tubulin used for these experiments was a different preparation from that used for the experiments shown in Table I. N.S., Not Significant.

<table>
<thead>
<tr>
<th>% of GDP:GTP (Total 1 mM)</th>
<th>Control</th>
<th>+MAP200</th>
</tr>
</thead>
<tbody>
<tr>
<td>0:100</td>
<td>(n = 18, 78.4 min)</td>
<td>(n = 30, 92.1 min)</td>
</tr>
<tr>
<td>25:75</td>
<td>(n = 18, 107.5 min)</td>
<td>(n = 19, 86.2 min)</td>
</tr>
<tr>
<td>50:50</td>
<td>(n = 31, 76.9 min)</td>
<td>(n = 23, 99.3 min)</td>
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Growth rate

<table>
<thead>
<tr>
<th>μm/min</th>
<th>% (100)</th>
<th>μm/min</th>
<th>% (100)</th>
</tr>
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<tbody>
<tr>
<td>1.37 ± 0.38</td>
<td>(68.1)</td>
<td>0.93 ± 0.30</td>
<td>(57.5)</td>
</tr>
<tr>
<td>0.79 ± 0.23</td>
<td>(47.5)</td>
<td>1.59 ± 0.46**</td>
<td>(78.7)</td>
</tr>
<tr>
<td>1.25 ± 0.28**</td>
<td>(72.8)</td>
<td>1.16 ± 0.51**</td>
<td>(72.8)</td>
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Shorting rate

<table>
<thead>
<tr>
<th>μm/min</th>
<th>% (100)</th>
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<tr>
<td>27.2 ± 7.9</td>
<td>(171.8)</td>
</tr>
<tr>
<td>23.1 ± 6.4</td>
<td>(288)</td>
</tr>
<tr>
<td>25.9 ± 9.5</td>
<td>(111.5)</td>
</tr>
<tr>
<td>25.5 ± 9.5N.S.</td>
<td>(148.2)</td>
</tr>
<tr>
<td>26.7 ± 11.4N.S.</td>
<td>(109.2)</td>
</tr>
<tr>
<td>25.3 ± 10.9N.S.</td>
<td>(109.2)</td>
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Catastrophe frequency

<table>
<thead>
<tr>
<th>N/min</th>
<th>% (100)</th>
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<tr>
<td>0.105 ± 0.008</td>
<td>(17.4)</td>
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<tr>
<td>0.181 ± 0.009</td>
<td>(117.8)</td>
</tr>
<tr>
<td>0.303 ± 0.009</td>
<td>(288)</td>
</tr>
<tr>
<td>0.140 ± 0.002*</td>
<td>(111.5)</td>
</tr>
<tr>
<td>0.156 ± 0.009*</td>
<td>(148.2)</td>
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<tr>
<td>0.207 ± 0.015*</td>
<td>(148.2)</td>
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Rescue frequency

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<tr>
<th>N/min</th>
<th>% (100)</th>
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<tr>
<td>1.09 ± 0.12</td>
<td>(17.4)</td>
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<tr>
<td>0.85 ± 0.11</td>
<td>(77.4)</td>
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<tr>
<td>0.67 ± 0.11</td>
<td>(60.9)</td>
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<tr>
<td>1.45 ± 0.11*</td>
<td>(46.5)</td>
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<tr>
<td>0.68 ± 0.16N.S.</td>
<td>(46.5)</td>
</tr>
<tr>
<td>0.50 ± 0.17N.S.</td>
<td>(34.4)</td>
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We compared the amount of coil formation when MAP200 was mixed with preformed taxol-stabilized microtubules (sample 1) to when taxol was mixed with preformed MAP200-tubulin complexes (sample 3). Simultaneously mixing tubulin, MAP200, and taxol served as an intermediate (sample 2). To score coil formation, we took advantage of the fact that microtubules pellet with centrifugation at 140,000 g whereas 390,000 g is required to pellet the coiled structures. Finding MAP200 enriched in the supernatant following the lower speed spin relative to the amount in the higher speed spin indicates enrichment of coiled structures. Note that if MAP200-tubulin complexes were unable to polymerize at all then the proteins would remain in the higher speed spin supernatant. It can be seen that treating MAP200-tubulin complexes with taxol (sample 3) gave rise to more coiled structures than when MAP200 was allowed to interact with microtubules (sample 1). This is inconsistent with the coiled structures representing depolymerization forms (e.g. ram’s horns; Mandelkow et al., 1991) and implies instead that taxol drives MAP200-tubulin complexes into long, protofilament-like polymers.

DISCUSSION

Comparison of MAP215 Activity among Eukaryotes

We characterized the activity of a plant MAP215 family member, tobacco MAP200, on microtubule dynamics in vitro. The results allow us to compare activities of MAP215 proteins from plants, animals, and fungi. In the presence of sufficient GTP, tobacco MAP200 influences microtubule dynamics similarly, but less strongly, than do the orthologs. MAP200 promotes growth rate, catastrophe, and rescue by about 1.4-fold, and has no effect on shortening rate (Table I); under similar conditions, X. laevis MAP215 promotes growth rate 8-fold, shorting rate nearly 3-fold, catastrophe nearly 2-fold, and all but eliminates rescue (Vasquez et al., 1994). Although in early experiments, XMAP215 suppressed catastrophe (Kinoshita et al., 2001), more recent experiments have confirmed that XMAP215 strongly promotes catastrophes (Shirasu-Hiza et al., 2003; Brouhard et al., 2008). The MAP215 ortholog in budding yeast, stu2p, also appears to be more active than MAP200, promoting catastrophe by 3.5-fold, shorting rate by 1.3-fold, and cutting growth rate in half (van Breugel et al., 2003). The promotion of catastrophe frequency appears to be highly conserved throughout the eukaryotic MAP215 family.

Plant MAP215 family members are close, in sequence and size, to their animal orthologs. Consistent with the high similarity, plant and animal MAP215 proteins promote both growth rate and catastrophe frequency in vitro. On the other hand, MAP200 increases rescue frequency whereas XMAP215 inhibits it (Vasquez et al., 1994). Interestingly, when GDP partially replaces GTP, MAP200 strongly curtails rescues,
which might indicate a similar activity for both X. laevis and tobacco orthologs. As an additional difference, we found that MAP200 fails to depolymerize GMPCPP-stabilized microtubules (data not shown) in the same buffer system where XMAP215 succeeds (Shirasu-Hiza et al., 2003; Brouhard et al., 2008). Taken together it appears that tobacco MAP200 is generally similar to animal MAP215 family members although orthologs from each organism differ in detail.

On the Mechanism of MAP215-Induced Catastrophe Induction

MAP215 orthologs, including MAP200, are able to increase both growth rate and catastrophe frequency. This is unusual because increased catastrophe is expected to be associated with microtubule shrinkage. To resolve this paradox, Brouhard et al. (2008) hypothesized that XMAP215 incorporates and removes tubulin dimers at the microtubule plus end by the same pathway, which stabilizes the dimer at an intermediate state between incorporation and dissociation. While this hypothesis explains the ability of the protein to increase both growth rate and catastrophe frequency, it does not predict the suppression of MAP200-induced catastrophe by GDP reported here (Table II; Fig. 2). Lessening catastrophe by adding GDP is curious because, according to the GTP-cap model, increasing concentrations of GDP-tubulin are expected to increase catastrophes (Erickson and O’Brien, 1992).

We put forward a new hypothesis to explain the behavior in MAP215 orthologs that is based on stress-induced catastrophe (Fig. 5). This hypothesis offers a plausible mechanism for the concomitant stimulation of growth rate and catastrophe as well as for MAP200’s suppressing catastrophe induced by GDP. Beginning with the assumption that MAP215 drives the formation of long protofilaments, we can distinguish two situations: When protofilaments occupy adjacent sites on the microtubule lattice, rapid and substantial growth would be sustained (Fig. 5A, a and b). However, when long protofilaments form at non-adjacent sites, their distal ends would tend to form strong, lateral contacts, as occur between adjacent protofilaments (Fig. 5A, c and d). Such a microtubule with misaligned protofilaments is prone to undergo catastrophe insofar as lattice arrangements other than the usual 13/3 helix contain excess free energy (Chretien and Fuller, 2000; Hunyadi et al., 2005). Thus, we hypothesize that in the presence of sufficient GTP, MAP215 enhances microtubule growth through driving protofilament extension and increases catastrophes through occasional, promiscuous lateral attachments between protofilaments. Although MAP200 and GTP-tubulin might form some other structure that induces catastrophe, such a structure is at present unknown.

The hypothesis can also account for the suppression of catastrophes under increasing GDP-to-GTP ratios because the opportunity for incorrect lateral contacts depends on rapid protofilament growth, which is arguably acutely sensitive to the presence of GDP tubulin. As the ratio of GDP-to-GTP tubulin increases, protofilament elongation will no longer be extreme, which will curtail MAP215-induced catastrophes, while the ability of MAP215 to extend protofilaments will continue to support microtubule growth (Fig. 5B).

**MAP215 and Microtubule Growth**

Two models have emerged for the mechanism whereby MAP215 family members drive tubulin incorporation (Asbury, 2008). The most recent model is surfing: This model posits that MAP215 remains at microtubule ends (much as a surfer rides a wave) and promotes insertion of tubulin dimers (Brouhard et al., 2008). These authors observed processive behavior of single GFP-XMAP215, adding up to 25 tubulin dimers at the microtubule end. In this model, there is only a one-to-one complex formation between XMAP215 and tubulin.

In contrast, the older, template model posits that MAP215 delivers multiple dimers to microtubule ends (Gard and Kirschner, 1987). This model is supported by observations with electron microscopy, showing that XMAP215 forms complexes with several tubulin dimers (Cassimeris et al., 2001) and by experiments with optical tweezers, reporting that XMAP215 drives microtubule growth in 60-nm steps, consistent with estimates of the size of the XMAP215-tubulin complex (Kerssemakers et al., 2006); however, this interpretation has been criticized (Schek et al., 2007). The

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**Figure 5.** Model for effects of MAP200 based on stress-induced catastrophe. A, In sufficient GTP, MAP200 forms an elongated protofilament that (a and b) induces a sheet-like structure favoring microtubule growth. Sometimes (c and d), two protofilaments elongate independently, which are nevertheless able to form distal lateral contacts. B, When GTP is limiting, MAP200-stimulated protofilaments are short. They are able to promote elongation modestly but unable to form undesired lateral contacts and hence unable to induce catastrophe.
template model is also supported by the amino acid sequence. Animal MAP215 family members have multiple (three to five) TOG domains (Slep and Vale, 2007), and a single TOG domain, albeit from budding yeast, was reported to bind a single tubulin dimer (Al-Bassam et al., 2007) although other work reported that two TOG domains are required per dimer (Slep and Vale, 2007). The sequence of MAP200 contains four TOG domains (Gard et al., 2004) and we show here that one MAP200 molecule binds four tubulin dimers, a stoichiometry consistent with the template model.

Despite uncertainty of how MAP215 favors tubulin incorporation, there is consensus that this protein drives protofilament extension, based on experimental and theoretical work (Vasquez et al., 1994; VanBuren et al., 2005; Brouhard et al., 2008). That tobacco MAP200 behaves similarly is supported by the formation, when microtubules polymerize in the presence of MAP200 and taxol, of coils with striking resemblance to paired, elongated protofilaments (Fig. 4, A and B). A key prediction of our hypothesis is that microtubules elongating in the presence of MAP215 will have long and sometimes entangled protofilament extensions. It should be possible to test this with cryo-electron microscopy. Such structural studies can help resolve the behavior of this enigmatic and essential microtubule-associated protein.

On the Suppression of Rescue

It is also noteworthy that although MAP200 promotes rescue in the presence of sufficient GTP, the promotion is lost as GDP is introduced (Tables I and II). Accounting for the basis of rescue events, Dimitrov et al. (2008) reported they occur at the sites of remnant GTP-tubulin within the microtubule lattice, such that rescue frequency is proportional to the frequency of unhydrolyzed GTP-tubulin. Combining this account with the template model for MAP215 activity and with our results that MAP200 forms a complex with either GTP-tubulin or GDP-tubulin (Fig. 3), we suggest that the differential effects of MAP200 on rescue in the absence or presence of GTP are explained by the nature of the MAP200 complex. For example, were MAP200 to have a higher affinity for GDP-tubulin than GTP-tubulin, then, as the concentration of GDP increased, complexes would be progressively enriched in GDP-tubulin subunits, and consequently, microtubules would grow with progressively fewer subunits containing GTP-tubulin delivered by MAP200, and thus fewer sites available for rescue. Measurement of these affinities as well as of the effects of mixed-subunit complexes on dynamic instability parameters will be required to fully understand MAP215 activity.

MEASUREMENTS AND METHODS

Purification of MAP200 and Tubulin

MAP200 and tubulin were prepared from tobacco (Nicotiana tabacum) BY-2 miniprotoplasts according to Hamada et al. (2004). MAP200 concentration was achieved by two-step column chromatography. Tubulin and MAP200 were roughly fractionized using Hi-trap Q HP (GE Health Care Bioscience) by linear, 0 to 1 M NaCl gradient. The fractions containing MAP200 were collected and supplied to Resource Q (GE Health Care Bioscience) and eluted by a linear, 0.3 to 0.5 M NaCl gradient at 0.1 mL/min for 120 min. MAP200 was isolated and concentrated to 12 μM or higher. The concentrated MAP200 was dialyzed with PME buffer (100 mM PIPES-KOH, 1 mM MgCl₂, 1 mM EGTA, pH 7.0) for assay. BY-2 tubulin is not suitable for measurements of dynamic instability because the lengths of microtubules were shorter than that of microtubules prepared from porcine brain tubulin. Porcine brain tubulin was prepared according to Itoh et al. (1997).

Measurements of Microtubule Dynamic Instability and Tubulin Turbidity

Dynamic instability of microtubules was observed with a dark-field microscope (Olympus) at 25°C. Images of microtubules were taken at 5 s intervals for 470 s. To polymerize microtubules, the mixture of 50 μM porcine brain tubulin and 1 mM GTP was incubated at 37°C for 3 min. Subsequently, equal volumes of a mixture of 0 to 8 μM MAP200 and 1 mM GTP were incubated at 37°C for 1.5 min and then added to microtubules. Final samples contained 25 μM tubulin, 1 mM GTP, and 0 to 4 μM MAP200. To examine the effect of GDP, the final ratio of GDP to GTP was controlled by adding a mixture of 4 μM MAP200 and various GDP:GTP concentrations to the reaction. Growth rate and shortening rate were averaged over the average of individual observed microtubules. Catastrophe frequency was calculated from total number of catastrophe events during total observation time. Rescue frequency was calculated from total number of rescue events during total catastrophe observation time. Data presented for dynamic instability parameters were taken from one experiment except for frequencies of catastrophe and rescue, which were obtained from three replicate experiments. In each experiment, approximately 20 microtubules were observed over a total of about 100 min. Significance levels were tested by t tests.

The turbidity of a tubulin solution (30 μM tubulin in PME buffer) was measured with a spectrophotometer (UV-2000, P/N206-17000, Shimadzu) in the absorbance measurement mode at 350 nm, 30°C. To assay the effect of MAP200, purified MAP200 was mixed with the tubulin solution on ice followed by GDP/GTP as indicated, with turbidity measurement starting immediately thereafter.

Assay of MAP200-GDP-Tubulin Complex and Coiled Structures

To prepare GDP-tubulin, microtubules were assembled in tubulin solution (30 μM tubulin in PME buffer) with GTP and harvested by centrifugation at 150,000 × g for 10 min at 30°C. The pellet of microtubules was disassembled by addition of 1 mM GDP on ice for 30 min, and then the sample was centrifuged at 150,000 × g for 10 min at 4°C. The supernatant of the sample was used as GDP-tubulin.

To prepare MAP200-GDP-tubulin complexes, MAP200 and GDP-tubulin were mixed in PME buffer with 1 mM GDP and incubated for 10 min at 30°C. The mixture of MAP200 and GDP-tubulin was subjected to negative staining with 2% uranyl acetate, and observed with an electron microscope (JEM-1200EXII, JEOL Ltd.). For Suc density gradient centrifugation, the mixture of MAP200 and GDP-tubulin was loaded on a 10% to 30% linear Suc gradient and centrifuged at 120,000 g for 12 h, 4°C. Each fraction was subjected to SDS-PAGE and stained by Coomassie Brilliant Blue. The ratio between MAP200 and tubulin dimer was calculated from the density of Coomassie-stained gels using Image J (http://rsb.info.nih.gov/ij).
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140,000q for 5 min or at 390,000q for 10 min. Supernatants and pellets were subjected to SDS-PAGE and stained by Coomassie.

Supplemental Data
The following materials are available in the online version of this article.
Supplemental Figure S1. Purified MAP200.
Supplemental Figure S2. Microtubule kymographs, extracted from some sequences used for Table I.
Supplemental Figure S3. Microtubule kymographs, extracted from some sequences used for Table II.
Supplemental Movie S1. Effect of MAP200 on dynamic instability.
Supplemental Movie S2. Effect of MAP200 on dynamic instability in the presence of 1 mM GTP.
Supplemental Movie S3. Effect of MAP200 on dynamic instability in the presence of 0.25 mM GDP and 0.75 mM GTP.
Supplemental Movie S4. Effect of MAP200 on dynamic instability in the presence of 0.5 mM GTP and 0.5 mM GTP.

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