Identification of XMAP215 as a microtubule-destabilizing factor in Xenopus egg extract by biochemical purification

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Microtubules (MTs) polymerized with GMPCPP, a slowly hydrolyzable GTP analogue, are stable in buffer but are rapidly depolymerized in Xenopus egg extracts. This depolymerization is independent of three previously identified MT destabilizers (Op18, katanin, and XKCM1/KinI). We purified the factor responsible for this novel depolymerizing activity using biochemical fractionation and a visual activity assay and identified it as XMAP215, previously identified as a prominent MT growth-promoting protein in Xenopus extracts. Consistent with the purification results, we find that XMAP215 is necessary for GMPCPP-MT destabilization in extracts and that recombinant full-length XMAP215 as well as an NH2-terminal fragment have depolymerizing activity in vitro. Stimulation of depolymerization is specific for the MT plus end. These results provide evidence for a robust MT-destabilizing activity intrinsic to this microtubule-associated protein and suggest that destabilization may be part of its essential biochemical functions. We propose that the substrate in our assay, GMPCPP-stabilized MTs, serves as a model for the pause state of MT ends and that the multiple activities of XMAP215 are unified by a mechanism of antagonizing MT pauses.

Introduction

Microtubule (MT)* polymerization dynamics have been implicated in many important cellular events, including cell polarization, motility, and division. They are regulated by cellular factors that both stabilize and destabilize the MT lattice. During mitosis, for example, increased MT dynamicity allows more efficient search and capture of kinetochores by MT ends (Holy and Leibler, 1994). This increased dynamicity is driven by increases in catastrophe rate in some systems (Belmont et al., 1990) and/or decreases in rescue rate in others (Glikman et al., 1992; Rusan et al., 2001). A number of MT dynamics regulators have been identified and characterized in recent years, and investigators in the field are actively pursuing the assignment of specific regulators to specific cellular events.

Three important MT destabilizers have been characterized in meiotic Xenopus egg extract: katanin (McNally and Vale, 1993), Op18/stathmin (Belmont and Mitchison, 1996), and XKCM1/MCAK (a member of the KinI family of kinesins) (Walczak et al., 1996). Of these three, the KinI family members appear to be the most important negative regulators of MT polymerization during mitosis (Belmont and Mitchison, 1996; Maney et al., 2001; Kline-Smith and Walczak, 2002). We set out to determine if there were any other MT destabilizers in Xenopus egg extract, using GMPCPP-stabilized MTs (CPP MTs) as the substrate in our depolymerization assays. CPP MTs were used in part for practical reasons (they are stable to dilution in buffer) and in part because they provide a novel assay that might identify factors with new mechanisms of action.

CPP MTs are stable to dilution because the nucleotide is only slowly hydrolyzed and thus mimics the GTP- or GDP-Pi-bound state (Hyman et al., 1992). However, we do not know precisely what state of physiological MTs they most closely resemble. They have been hypothesized to mimic the GTP cap, a hypothetical structure stabilizing the ends of actively growing MTs (Drechsel and Kirschner, 1994; Caplow and Shanks, 1996). In this paper, we suggest an alternative possibility, that CPP MTs most closely mimic a hypothetical “paused” state of the MT lattice, an intermediate between the growing and shrinking states (Tran et al., 1997).

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Results

Meiotic Xenopus egg extracts contain a novel MT-depolymerizing factor

To assay for MT-depolymerizing factors, we added rhodamine-labeled CPP MTs to crude or clarified cytostatic factor (CSF)–arrested Xenopus egg extract (CSF extract) and observed their disappearance over time. CPP MTs are stable to dilution in buffer, but when added to extract, they depolymerize in 5–10 min (Caplow, M., personal communication). To characterize this depolymerizing activity, we sedimented clarified CSF extract on a 5–20% sucrose gradient and assayed fractions for depolymerizing activity. A single ATP-independent peak of activity was observed at ~9.5S (Fig. 1 B). XKCM1 cosedimented with this peak (Fig. 1 A), but katanin and Op18 did not (unpublished data). The activity appeared to be independent of XKCM1 because XKCM1 requires ATP for efficient MT depolymerization (Desai et al., 1999b). To confirm that XKCM1 was not responsible for the depolymerizing activity, we assayed those fractions in the absence of ATP and in the presence of inhibitory \(\alpha\)-XKCM1 antibody (Walczak et al., 1996) (Fig. 1 B). Depolymerizing activity was not blocked, suggesting that another factor was responsible.

Identification of the depolymerizing activity as a fragment of XMAP215

We purified the unknown CPP MT–depolymerizing factor using conventional chromatography. The assay consisted of adding rhodamine-labeled CPP MTs to each fraction and fixing at time points to observe the disappearance of these MTs by fluorescence microscopy. Relative activity of each fraction was estimated by serial dilution. The key strategic issue was separation of the novel CPP MT–depolymerizing activity from other activities that either inhibited the assay (bundling factors) or scored in the assay (known destabilizers). To avoid confusion between the novel activity and XKCM1, we purified the novel activity from a 40% ammonium sulfate (AS) supernatant of clarified CSF extract, which contained only a fraction of total depolymerizing activity, but was free of the known depolymerizers, XKCM1, katanin, and Op18, by Western blot (unpublished data).

The CPP MT–depolymerizing factor was purified using seven steps: AS precipitation, phenyl sepharose, heparin, monoS, gel filtration, monoQ (pH 7.2), and a final monoS column. When fractions were separated by SDS-PAGE and silver stained, a set of polypeptides of \(~130\) kD and a protein of \(~160\) kD consistently coeluted with activity on the last two columns in the purification (Fig. 2, A and B, arrows). We estimated that specific activity was enriched several thousand fold by the final monoS step (Table I).

Attempts at further purification resulted in extensive loss of activity and protein. Instead, two independent purifica-
tion steps (sucrose gradient and monoQ, pH 8.8) were performed in parallel with the above purification, starting with active fractions from the gel filtration step. In both of these steps, the same set of 130- and 160-kD proteins continued to peak in active fractions (unpublished data).

The cluster of polypeptides at 130 kD were excised from an 8% polyacrylamide gel and identified by liquid chromatography tandem mass spectrometry. 21 peptides from the tryptic digest matched the sequence of XMAP215, a previously identified 215-kD *Xenopus* MT-binding protein (Gard and Kirschner, 1987). Each of these peptides mapped to the NH2-terminal half of the sequence, suggesting that we had purified an NH2-terminal fragment of XMAP215 (unpublished data). Western blots using antibodies specific to the NH2- and COOH termini of XMAP215 (Fig. 2 C) confirmed that the set of p130 bands as well as the p160 band enriched in our purification were NH2-terminal fragments of XMAP215.

XMAP215 is a major CPP MT–depolymerizing factor in *Xenopus* egg extract

We next investigated whether XMAP215 constituted a CPP MT–depolymerizing factor in CSF extracts. Though we had purified a set of NH2-terminal XMAP215 fragments from crude extract, we could not detect those fragments by Western blot in crude or clarified extract. XMAP215 appeared to exist as a full-length 215-kD species. This full-length XMAP215 comigrated with the 9.5S peak of depolymerizing activity we originally observed during sucrose gradient
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represented by the top, middle, and bottom of each box. expressed as percent median value of buffer control; error bars denote 10th and 90th percentile; the 75th, 50th, and 25th percentiles are terminal (C) XMAP215. MT polymer is able to deplete efficiently, and NH2-terminal fragment (N), or a COOH-terminal fragment (C). Shown here are representative fluorescence images for four concentrations of each protein. Bar, 10 μm. (B) Full-length XMAP215 and the NH2-terminal fragment have depolymerizing activity between 6.25 and 200 nM. MT polymer was quantitated for each sample by calculating average fluorescent pixel area per field for each protein concentration of full-length (F), NH2-terminal (N), and COOH-terminal (C) XMAP215. MT polymer is expressed as percent median value of buffer control; error bars denote 10th and 90th percentile; the 75th, 50th, and 25th percentiles are represented by the top, middle, and bottom of each box.

Figure 4. Pure recombinant XMAP215 depolymerizes CPP MTs in vitro. (A) Full-length XMAP215 and an NH2-terminal fragment of XMAP215 both depolymerize CPP MTs, but a COOH-terminal fragment does not. Rhodamine-labeled CPP MTs were incubated for 15 min in buffer containing different concentrations of full-length XMAP215 (F), an NH2-terminal fragment (N), or a COOH-terminal fragment (C). Shown here are representative fluorescence images for four concentrations of each protein. Bar, 10 μm. (B) Full-length XMAP215 and the NH2-terminal fragment have depolymerizing activity between 6.25 and 200 nM. MT polymer was quantitated for each sample by calculating average fluorescent pixel area per field for each protein concentration of full-length (F), NH2-terminal (N), and COOH-terminal (C) XMAP215. MT polymer is expressed as percent median value of buffer control; error bars denote 10th and 90th percentile; the 75th, 50th, and 25th percentiles are represented by the top, middle, and bottom of each box.

XMAP215 depolymerizes CPP MTs in vitro

We next assayed pure, baculovirus-expressed XMAP215 for CPP MT–depolymerizing activity in vitro, using both full-length and truncated XMAP215 constructs previously characterized by Popov et al. (2001; see Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200211095/DC1). Both full-length protein and an NH2-terminal fragment (aa 1–560) were able to depolymerize rhodamine-labeled CPP MTs in vitro (Fig. 4 A). In serial titrations, activity for both polypeptides was similar and measurable, beginning between 6.25 and 12.5 nM (Fig. 4 B). The full-length protein sample does contain a small amount of cleaved protein, so we cannot definitively rule out that this is not the active species in our assay; however, the majority of the protein is full-length. The NH2-terminal fragment does not appear to be significantly more potent than the full-length protein. A COOH-terminal fragment of XMAP215 (aa 1168–2065), on the other hand, was completely inactive in the depolymerization assay (Fig. 4, A and B). We measured depolymerizing activity in the visual assay by using fluorescent pixel area per visual field to quantitate MT polymer. Sedimentation assays and quantitation of tubulin in supernatants and pellets gave similar results (unpublished data). Samples with high concentrations of full-length XMAP215 (stoichiometric with tubulin, ~200 nM) showed less depolymerization and highly bundled MTs (Fig. 4 A). This was also seen, to a lesser ex-
tent, in samples with very high concentrations of NH₂-terminal fragment (unpublished data). The COOH-terminal fragment did not cause bundling at any concentration.

It is not surprising that we do not normally see the polymerization-promoting activity of XMAP215 in our assay, even at concentrations similar to previously published reports. Our assay differs from dynamic MT assays in several ways that strongly select for depolymerizing activity: the assay is performed at room temperature, uses CSF-XB instead of BRB80 buffer, and contains little or no soluble tubulin dimer for polymerization. The same full-length XMAP215 construct used in our depolymerization assays was shown to have polymerization-promoting activity in dynamic MT assays (Kinoshita et al., 2001).

**XMAP215-promoted depolymerization is specific to MT plus ends**

To test if XMAP215 promotes CPP MT depolymerization by an end-dependent mechanism, we recorded depolymerization live in glass flow-cells using time-lapse fluorescence microscopy. Rhodamine-labeled CPP MTs were bound to glass using kinesin and then treated with buffer or buffer containing XMAP215. In buffer alone, MTs were relatively stable for 30 min; in the presence of 19 nM XMAP215, they depolymerized over several minutes in an endwise fashion (Fig. 5A). There was a strong polarity bias to depolymerization. We used dim-bright CPP MTs and kinesin motility to determine that XMAP215 depolymerized the MT plus end at a rate 5–10 times faster than buffer alone, whereas minus end depolymerization was not measurably affected (Fig. 5B). In the presence of XMAP215, 92 out of 95 MTs (96.8%) had faster rates of depolymerization on their lagging (plus) ends than on their leading (minus) ends. Thus, XMAP215 specifically promotes CPP MT depolymerization at plus ends (see Video 1, available at http://www.jcb.org/cgi/content/full/jcb.200211095/DC1). Its polymerization-promoting activity is also plus end specific (Gard and Kirschner, 1987; Vasquez et al., 1994).

**Mechanism of CPP MT depolymerization by XMAP215**

There are several mechanisms by which XMAP215 might accelerate CPP MT depolymerization. XMAP215 might sequester GMPCPP-tubulin dimers, increase the rate of GMPCPP hydrolysis, or increase the dissociation rate by disrupting the lattice. We ruled out the trivial possibility that our XMAP215 protein preparation contained proteolytic activity by performing SDS-PAGE of depolymerization products (unpublished data).

To test if dimer sequestration accelerates apparent CPP MT depolymerization (by inhibiting readdition of subunits to MT ends), we added nocodazole to CPP MTs diluted in buffer alone (Fig. 6A). The same concentration of nocodazole added before CPP MT polymerization completely inhibited polymerization (unpublished data). However, this potent monomer-sequestering drug did not stimulate depolymerization of CPP MTs in our assay, presumably because the total tubulin concentration is too low to allow significant readdition of dimer to MT ends. To test if GMPCPP was hydrolyzed during XMAP215-promoted depolymerization, we used MTs polymerized with [γ-32P]GMPCPP and separated from unbound nucleotides by sedimentation through a sucrose cushion. No hydrolysis was observed in buffer or XMAP215, though Na-BRB80/60% glycerol (a positive control; Caplow et al., 1994) did stimulate hydrolysis (Fig. 6B).

To test if XMAP215 disrupted protofilament interactions within the MT lattice, we imaged CPP MTs before and during treatment with either full-length or NH₂-terminal XMAP215 by negative-stain electron microscopy. In buffer alone, CPP MT ends were blunt (Fig. 6C). In the presence of XMAP215 (full length or NH₂-terminal fragment), we consistently observed bulbs of material at the ends of the depolymerizing MTs. In some images, these bulbs appeared to contain curled up protofilaments. We cannot state definitively if these structures contain tubulin, XMAP215, or both; however, they are reminiscent of those resulting from treatment with KinI kinesins (Desai et al., 1999b). We favor a similar unpeeling mechanism for CPP MT depolymerization by XMAP215, though KinI and XMAP215 do differ mechanistically in two interesting
polymization of CPP MTs in vitro. XMAP215 had not previously been tested for its ability to depolymerize CPP MTs, an artificial MT substrate. We believe that this in vitro activity could have important mechanistic implications for both the molecular mechanism of XMAP215 and, more broadly, the mechanism of dynamic instability. Before discussing those mechanistic implications, we will first briefly discuss the physiological significance of our results.

Finding that XMAP215 is a major MT-destabilizing factor is at odds with the current view of this protein as an important MT growth–promoting factor. It was first discovered more than 10 yr ago by Gard and Kirschner (1987), through biochemical fractionation and a visual assay for MT polymerization, almost the converse of our depolymerization assay. Homologues exist in almost every organism, including Saccharomyces cerevisiae (stu2), Schizosaccharomyces pombe (dis1, alp14), Caenorhabditis elegans (zyg-9), Drosophila melanogaster (msps), Arabidopsis (mor1), and humans (ch-TOG) (for review see Ohkura et al., 2001). The two most common phenotypes for decreased levels of this protein family are short MTs and defects in spindle pole formation. In vitro, pure XMAP215 is known to promote polymerization specifically on the MT plus end (Gard and Kirschner, 1987; Vasquez et al., 1994), and careful combination of brain tubulin, XKCM1, and XMAP215 can recapitulate nearly physiological levels of all four parameters of dynamic instability (Kinoshita et al., 2001). Together, these in vivo and in vitro data have led to the model that members of the Dis1/XMAP215 family are important factors regulating physiological MT dynamics in all cells by promoting polymerization.

In light of our results, it will be interesting to investigate more closely whether Dis1/XMAP215 family members might also play a role in MT depolymerization in the cell. Consistent with this, recent work by van Breugel et al. (2003) demonstrates that the S. cerevisiae homologue (Stu2) does not promote MT growth in vitro but instead slows polymerization and promotes catastrophes. There are at least two places where Dis1/XMAP215 family members are candidates for site-specific depolymerizing activity. First, in fission yeast, both homologues (Dis1 and Alp14) localize to kinetochores, which are sites for plus end depolymerization (as well as polymerization) during chromosome oscillation and segregation (Garcia et al., 2001; Nakaseko et al., 2001). Tantalizingly, recent evidence in that system points to a synergistic, not antagonistic, relationship between Dis1/Alp14 and the K11-like kinesins klp5/6 at the kinetochore (Garcia et al., 2002). Second, in every system examined to date, Dis1/XMAP215 localizes tightly to centrosomes and mitotic spindle poles (Ohkura et al., 2001). An MT-depolymerizing factor that localizes to spindle poles would be an attractive candidate for the minus end–depolymerizing activity associated with poleward MT flux. The tiny spindles that result from XMAP215 depletion in frog extract have not been tested for their flux rates. However, at least in vitro, XMAP215 depolymerization appears to be specific to MT plus ends. It is possible that XMAP215 acts as an MT polymerizer at centrosomes and an MT depolymerizer at kinetochores. Or, it is possible that XMAP215 at centrosomes depolymerizes MTs that are misoriented with their minus ends

Discussion

XMAP215 is a major CPP MT–depolymerizing factor in Xenopus egg extract

MT dynamics are subject to regulation by both stabilizing and destabilizing factors in vivo. Our overall goal in this project was to identify and characterize novel destabilizers. Although three such factors were already known in Xenopus egg extracts (Op18/stathmin, XKCM1/Kin1, and katanin), our initial experiments with CPP MTs suggested that these factors could not account for all destabilizing activity. We set out to isolate the novel factor(s) using biochemical fractionation and purified a fragment of XMAP215. We subsequently showed that XMAP215 is a major CPP MT–depolymerizing factor in Xenopus egg extract and that low concentrations of pure recombinant XMAP215 promote depolymerization of CPP MTs in vitro. The Journal of Cell Biology | Volume 161, Number 2, 2003
out or, at spindle poles, depolymerizes spurious plus ends from the opposite pole. Interestingly, the major phenotype of decreasing ch-TOG levels in HeLa cells by RNAi is not MT destabilization (as would be expected for an MT stabilizer) but spindle MT disorganization (Gergely et al., 2003). Further investigation will be necessary to determine if XMAP215 ever functions in vivo as an overt depolymerizer.

**Mechanistic implications for XMAP215**

Although most of the literature focuses on the ability of XMAP215 to promote polymerization, our observation that XMAP215 can destabilize MTs is not without precedent. Vasquez et al. (1994) had previously shown that purified XMAP215 increased the MT depolymerization rate as well as polymerization rate, and that it inhibits rescue events. These data are consistent with lattice-destabilizing activity, as are the data of van Breugel et al. (2003) for Stu2.

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Because full-length XMAP215 had depolymerizing activity in our in vitro assay, proteolysis cannot account for conversion of a polymerizing factor into a depolymerizing factor. Both polymerizing activity (Popov et al., 2001) and depolymerizing activity map roughly to the NH2-terminal 1/4 of XMAP215. More precise mapping might separate these functions in the future. However, given that both activities act primarily on MT plus ends, our current working model is that XMAP215’s polymerization-promoting and CPP MT–destabilizing activities are two aspects of a common biochemical mechanism.

What might this common mechanism be? Our preliminary studies suggest a model in which XMAP215 alters the conformation of the MT end to promote depolymerization, possibly by affecting interactions between protofilaments. The mechanism by which XMAP215 promotes polymerization specifically on the MT plus end is not known. Two hypotheses have been considered (Spittle et al., 2000): XMAP215 might oligomerize tubulin dimers in solution and thus catalyze addition of several dimers per association event; alternatively, it might alter the structure of the growing end, promoting a structure that either adds dimers more rapidly or is less likely to undergo brief pause events. The latter model, in which Dis1/XMAP215 modifies the end of the MT lattice so as to promote dynamicity, potentially allows a unified explanation for all four activities of the protein (promoting polymerization, promoting depolymerization, antagonizing/inhibiting rescue, and depolymerizing CPP MTs). A key clue might come from the specialized, nonphysiological CPP MT substrate and in understanding what physiological state it mimics most closely.

The CPP lattice has most often been used as a model for the GTP cap (Drechsel and Kirschner, 1994; Caplow and Shanks, 1996). However, the blunt-ended, closed tube structure of the CPP MT lattice is not similar to the sheet-like end of a growing MT, nor does it resemble the rams’ horns of a shrinking MT (Simon and Salmon, 1990; Mandelkow et al., 1991; Chretien et al., 1995). We propose instead that CPP MTs are a model for the MT pause state. Tran et al. (1997) proposed a three-state model for dynamic instability in which the pause state is an obligate intermediate between polymerization and depolymerization. Neither the structure nor the bound nucleotide of the hypothetical pause state is known. It seems reasonable to suggest that the pause state might have a blunt-ended, closed tube structure, intermediate between the sheet-like protofilament extensions and curled protofilaments characteristic of growth and shrinkage. Consistent with this idea, Chretien et al. (1995) proposed that loss of sheet-like protofilament extensions correlated with slower growth. A plus end that paused long enough would presumably exchange nucleotide at the exposed E-sites (Mitchison, 1993), resulting in GTP-bound tubulin subunits at the tip of a paused plus end. The exposed end of a CPP MT that is blunt and contains a GTP analogue may mimic this hypothetical blunt, exchanged state of an MT in which all the internal subunits are GDP bound.

This interpretation of what the CPP lattice mimics prompts us to propose that XMAP215 destabilizes the pause state, acting as an antipause factor (Fig. 7). MTs frequently pause in vivo, spending prolonged time neither growing nor shrinking at the resolution level of the light microscope (Shelden and Wadsworth, 1993; Tirmauer et al., 1999; Rusan et al., 2001). MTs also pause during phases of polymerization and depolymerization in Xenopus extracts (Tirmauer et al., 2002). Pauses are infrequent in reports of pure tubulin dynamics (Walker et al., 1988), but it is possible that pure MTs undergo micropauses too short to be detected by conventional imaging. In this pause state, MTs can theoretically transition into either growth or shrinkage, and a factor that destabilizes the pause state would increase MT dynamicity. Whether the MT transits to growth or shrinkage may depend on its environmental cues (tubulin concentration, other proteins, nucleotides, salt, or buffer); this would ex-

![Figure 7](Image)
plain the apparently contradictory behavior of XMAP215 in different contexts. An antipause factor would also increase both apparent polymerization and depolymerization rates if polymerization and depolymerization were rate limited by microtubules. Higher resolution tracking of growing ends with pure tubulin could test this assumption. The antipause hypothesis could also account for the plus end specificity of XMAP215 if, for example, microtubules, corresponding to loss of protofilament extensions (Chretien et al., 1995), limit plus end growth and shrinkage more than minus end growth and shrinkage. Indeed, the pause model was introduced to account for different stabilities of the plus and minus ends (Tran et al., 1997).

MT depolymerization is necessary for every aspect of mitotic spindle function, from the breakdown of MTs in prophase to the search and capture of kinetochores to kinetochore oscillations, flux, anaphase movement, and spindle disassembly after anaphase. The in vivo function of Dis1/XMAP215, currently thought of as an MT growth–promoting factor, should be reexamined to look for functions that might depend on its MT-destabilizing activity, an equally important aspect of its biochemistry. Interesting areas of future research include determining whether the two apparently opposed activities of XMAP215 are separable, either biochemically or by mutation, and asking if XMAP215 exhibits these two activities because it is, fundamentally, an antipause factor. Addressing these questions should inform us as to the molecular mechanisms underlying physiological MT dynamics.

Materials and methods

Xenopus egg extracts

CSF-arrested extracts and in vitro spindle assembly reactions were prepared as previously described (Desai et al., 1999a). For large-scale purification, the following adjustments were made. Packing and crushing spins were performed in 30 ml (28.7 × 103.3 mm) tubes (Nalgene; Nunc), with 25–30 ml of eggs per tube. The packing spin consisted of 1 min at 500 rpm and 30 s at 1 kpm in a clinical centrifuge (Sorvall); the crushing spin was performed at 12.5 kpm (~21,000 g) for 15 min at 16°C in a SureSpin 630 rotor (Sorvall). Crude CSF extract was then clarified at 4°C either in a TH-641 rotor (Sorvall) for 4 h at 41 kpm (~200,000 g) or in two AH650 rotors (Sorvall) for 2 h at 50 kpm (~235,000 g). Typically, 20–25 ml of clarified CSF extract (~40 mg protein/ml) was obtained from 1 liter of eggs (predejelly stage). Clarified extract was supplemented with cyto D (10 μg/ml final; Sigma–Aldrich), protease inhibitors (10 μg/ml each final, leupeptin, pepstatin A, chymostatin; Sigma–Aldrich), and energy mix (7.5 mM creatine phosphate, 1 mM ATP, 1 mM GMP; final) before being flash frozen in liquid nitrogen in 1-ml aliquots.

In vitro assay for CPP MT depolymerization

Tubulin was labeled with tetramethyl- or X-rhodamine (Molecular Probes) in vitro assay for CPP MT depolymerization. When necessary, 100 μM EGTA, 2 mM MgCl₂, 0.1 mM CaCl₂). After 15 min, 3 μl of each reaction was mixed thoroughly with 3 μl of cold buffer (50 mM 1 mM DTT). For quantitative measurement of activity with pure XMAP215 constructs, the following adjustments were made. Assays were performed in extract buffer (CSF-XB, 100 mM KCl, 50 mM sucrose, 10 mM K-Hepes, pH 7.7, 5 mM EGTA, 2 mM MgCl₂, 0.1 mM CaCl₂). After 15 min, 3 μl of each reaction was mixed thoroughly with 3 μl of cold buffer (50 mM 1 mM DTT). When necessary, 100 μl of each fraction was desalted using 1-ml disposable spin columns filled with equilibrated G-25 fine resin. Desalting of samples with low protein concentration led to high loss of activity unless detergent was added (0.5% CHAPS) or protein concentration supplemented to 0.5 mg/ml with purified ovalbumin (Sigma–Aldrich).

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These fractions were pooled (monoS1), concentrated via microcon to a final volume of 100 μl, refiltered through a 0.22-μm spin filter, and applied to a 1-ml SMART system Superose 6 column (Amersham Biosciences) that had been previously equilibrated with assay buffer. Activity eluted at ~1.45 ml. Four or five fractions of 30 μl each were pooled (sup), diluted into MQ buffer (180 mM KCl, 20 mM Tris-HCl, pH 6.0, 50 mM sucrose, 5 mM MgCl2, 5 mM EGTA), and loaded on a 100-ml SMART system MonoQ column (Amersham Biosciences). Activity appeared in the flowthrough (Q FT), which was supplemented with MOPS to pH 7.0 and diluted to a final KCl concentration of 50 mM. The Q FT was applied to a 100-ml SMART system MonoS column (Amersham Biosciences). During a linear gradient of 0–50 mM KCl, a single peak of activity again eluted at 160 mM KCl. Sup and MonoS fractions (monoS2) were collected and analyzed. Fractions were pooled and sedimented on a 2-ml 5–20% sucrose gradient (TLS-55 rotor, 50 krpm, 4 h, 4°C) or run on SDS-PAGE for silver stain.

Purification was complicated by nonspecific losses in activity when protein concentration was too low. For this reason, in the last two or three steps, protein levels were supplemented to 0.5 mg/ml during column loading with purified ovalbumin, which binds monoQ and flows through monoS in our MQ and MS buffers, respectively. As losses in activity were also incurred by freeze-thaw, the purification protocol was performed over several days at 4°C, without freezing any active fractions.

**Mass spectrometry**

MonoS final fractions were separated on an 8% polyacrylamide gel by SDS-PAGE. The gel was silver stained with the following: 10 min in 1% SDS; 30 min in 20% methanol; 10 min in 5% methanol; 10 min in 1 M H2O containing 8 μl of 1 M DTT; 10 min in silver solution (0.2% AgNO3); brief wash with milliQ water; 3 min in silver solution (0.2% AgNO3); brief wash with a small amount of developing solution (7.5 g of Na2CO3 in 250 ml water plus 125 μl 37% formaldehyde); brief wash with a small amount of milliQ water; addition of the rehydrating solution until bands are of desired intensity; quench by pouring off developing solution and adding 5% AcOH; 3 × 15 min washes with water. After silver stain, pI30 bands were carefully excised and subjected to trypptic digest before liquid chromatography tandem mass spectrometry and database analysis; these procedures were performed at the Taplin Biological Mass Spectrometry Facility at Harvard Medical School.

**Immunoreagents**

Antibodies specific to the NH-terminal 360 aa of ch-TG and antibodies specific to the COOH-terminal 15 aa of ch-TG were a gift from K. Kinosita and A. Hyman. Anti-katanin antibody was a gift from F. McNally (University of California, Davis, CA). Inhibitory XKCM1 antibodies were provided by both C. Walczak (University of Indiana, Bloomington, IN) and R. Ohi (Harvard University) inhibitory activity was confirmed both in extract (dehyd; both C. Walczak (University of Indiana, Bloomington, IN) and R. Ohi (Harvard University) inhibitory activity was confirmed both in extract (dehyd) and in vitro assays with recombinant XKCM1 (not depicted). Immunodepletion of Xenopus egg extracts was performed with Dynabeads as previously described (Tournebize et al., 2000). Efficient depletion of XMAP215 was achieved using a polyclonal rabbit antibody raised against the last 16 aa at the COOH terminus after two rounds of depletion, using 12.5 μg of antibody per 50 μl of beads per round for 140 μl of crude extract. Similar concentrations of rabbit IgG (Sigma-Aldrich) and anti-XKCM1 antibody were used for each round of mock and XKCM1 depletions.

**Time-lapse microscopy and flow cell assay**

Flow cells were constructed using GoldSeal glass slides, 18 × 18-mm square GoldSeal coverslips, and thin strips of double-sided Scotch tape. Each coverslip was rinsed in acetone for 10–15 min before being spun dry and then air dried on Whatman paper (13–10 min). Coverslips were inverted onto two pieces of double-sided tape stuck to a glass slide, creating chambers of ~10–15 μl. Reagents were pipetted into one end and drawn out the other with triangles of whatman paper in this order: (1) 1 vol of 100 μg/ml kinesin (gift from Z. Maliga, Harvard University) in 20 mM Tris-HCl, pH 7.0, 1 mM DTT, incubated 10 min; (2) 5–8 vol of 6.5 mg/ml casein, incubated 10 min in BRB80; (3) 3–5 vol of 200 nM XMAP215 (full-length recombinant protein), incubated 10 min; (4) 3–5 vol of 200 nM XMAP215, usually diluted to 400 nM, incubated 10 min; (5) 5–8 vol BRB80 + 1 × oxygen scavenging mix (OS, 4.5 mg/ml glucose, 0.035 mg/ml catalase, 0.2 mg/ml glucose oxidase, 0.5% β-mercaptoethanol in CSF-XB); (6) 5–8 vol CSF-XB + OS; (7) 3–5 vol CSF-XB + OS + 19 mM XMAP215 (full-length recombinant protein) ± 10 μM GMPCPP. Though kinesin motility was fast and reliable in BRB80, CPP MTs were often released by kinesin in CSF-XB, and hence concentrations of kinesin in step 1 and low ATP concentrations in step 7. Dim-bright CPP MTs were made as previously described (Hyman, 1991), polymerizing 0.4 mg/ml of 1:l (labeled/unlabeled) tubulin plus 200 μM CPP for bright seeds and using 36 μg/ml of these seeds in ~0.2 mg/ml of 1:l (labeled/unlabeled) tubulin for dim MT elongation. Time-lapse movies were made by taking 100-ms exposures (bin = 2) every 5–15 s, using microscopy equipment as described above. Movies were analyzed using Metamorph as follows: movies were recorded as stacks; planes corresponding to two time points were duplicated from the stack; using color combine, the two planes were overlaid in two different colors; using the line region tool, MT lengths were measured on either side of a fiduciary mark; polarity could be assigned by comparing the location of the fiduciary mark in each plane. We only used MTs with clearly distinguishable ends in both planes and clear movement of the fiduciary mark. MT length measurements were logged to a spreadsheet in Microsoft Excel for further analysis.

**GMPCCP hydrolysis**

γ-32P-GMPCCP was synthesized from GMPCP and γ-32P-ATP, 2 μl of nucleotide diphosphate kinase (Sigma-Aldrich), 15 μl of 1 μM GMPCP in BRB80, and 15 μl of γ-32P-ATP were incubated at room temperature for 6 h. The reaction was spun for 15 min in a microfuge, and the supernatant was filtered through a 10K cut-off filter. 0.1 μl of each reaction product was analyzed by TLC using PEI-cellulose plates (Baker-Flex) run in 1.0 M LiCl and detected using a phosphorimager (Molecular Imager FX; Bio-Rad Laboratories) and Quantity One v.4.1.1 software. Standards (1 μl each of 10 mM GMPCCP, ATP stocks) were run in parallel and detected using a handheld UV lamp.

γ-32P-GMPCCP was used to monitor phosphate hydrolysis in the depolymerization reaction. Depolymerization reactions were performed as described above, using 75 nM full-length, recombinant XMAP215 or NH-terminal fragment, except that 15 μl of γ-32P-GMPCCP was added during CPP MT polymerization to incorporate it into the lattice. Reactions without γ-32P-GMPCCP were performed in parallel, to monitor the extent of depolymerization by visual assay. Phosphate hydrolysis was monitored by taking 6.7 μl of each reaction at 0, 10, and 20 min for assays in assay buffer and at 0, 30, and 60 min for assays performed in BRB80 or BRB80 + 5 mM EDTA. Results were equivalent for each buffer condition. Time points were quenched by addition of an equal volume of denaturing buffer (8 M urea, 20 mM Tris-HCl, pH 7.0, 5 mM EDTA). As a positive control, depolymerization reactions were performed in 60% glycerol/Na-BRB80, which is known to induce hydrolysis of GMPCCP. Free γ-32P was separated from γ-32P-GMPCCP by TLC on PEI-cellulose using 0.75 M sodium phosphate, pH 4.2, after first prewashing (postload) each TLC plate with ddH2O to get rid of excess salt, urea, and glycerol. Radioactive reaction products were detected using a Molecular Imager FX phosphorimagery and Quantity One v.4.1.1 software.

**Negative stain EM**

Negative stain EM was performed as previously described (Desai et al., 1999). Standard depolymerization reactions were performed, using 38.5 nM XMAP215, except that each sample was spun for 15 min on high at 4°C in a microfuge before addition of rhodamine-labeled CPP MTs, and reactions were performed in BRB80 buffer.

**Online supplemental material**

The supplemental material (Figs. S1 and S2; Video 1) is available at http://www.jcb.org/cgi/content/full/jcb.200211095/DC1. Fig. S1 shows representative fluorescent images of spindles made in extracts depleted with control (IgG), αXKCM1, or αXMAP215 antibodies. Fig. S2 is a Coomassie-stained gel of the three XMAP215 constructs used in vitro depolymerization assays. Video 1 is a time-lapse video of dim-bright microtubules treated first with buffer alone and then with buffer plus 19 μM XMAP215. The microtubules are being translocated with their minus ends leading.

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