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XKCM1 Acts on a Single Protofilament and Requires the C Terminus of Tubulin

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The stability of microtubules during the cell-cycle is regulated by a number of cellular factors, some of which stabilize microtubules and others that promote breakdown. XKCM1 is a kinesin-like protein that induces microtubule depolymerization and is required for mitotic spindle assembly. We have examined the binding and depolymerization effects of XKCM1 on different tubulin polymers in order to learn about its mechanism of action. Zinc-induced tubulin polymers, characterized by an antiparallel protofilament arrangement, are depolymerized by XKCM1, indicating that this enzyme acts on a single protofilament. GDP-tubulin rings, which correspond to the low-energy state of tubulin, are stable only under conditions that inhibit XKCM1 depolymerizing activity, but can be stabilized by XKCM1 bound to AMPPNP. Tubulin polymers made of subtilisin-treated tubulin (lacking the tubulin C-terminal tail) are resistant to XKCM1-induced depolymerization, suggesting that the interaction of the acidic tail of tubulin with basic residues in XKCM1 unique to Kin I proteins is required for depolymerization.

Keywords: XKCM1; Kin I kinesins; MTs; zinc macrotubes; GDP-tubulin rings

Introduction

Microtubules (MTs) are critical components of all eukaryotic cells. They are involved in a wide variety of cellular functions, ranging from segregation of genetic material during mitosis to organelle transport and cell movement. MTs are polymers of αβ-tubulin heterodimers that arrange in a head-to-tail fashion into protofilaments. Physiological MTs are 25 nm in diameter and typically contain 13 protofilaments that associate in a parallel fashion to form the hollow structure of the microtubule. Two characteristics of MTs are pivotal in their cellular function. First, due to the head-to-tail arrangement of tubulin heterodimers in parallel protofilaments, MTs are polar. This property enables motor proteins to use MTs as tracks for transport of cargo in a specific direction. Second, MTs, both *in vivo* and *in vitro*, exhibit dynamic instability, a behavior in which MTs coexist in states of growth and shrinkage and interconvert randomly between these two states.[1] Dynamic instability is driven by GTP hydrolysis on β-tubulin within the MT lattice. Polymerized GDP tubulin has a preferred curved conformation,[2,3] but within the microtubule lattice, it is thought to be kept straight by lateral contacts between the protofilaments.[4] Growing MTs contain a stabilizing cap of GTP-bound subunits that are thought to stabilize the microtubule ends. Upon loss of this GTP cap, protofilaments relax into their curved conformation and peel off,[5] resulting in rapid microtubule depolymerization.

Given the important role that microtubule dynamic instability plays in cytoskeletal function, this property is regulated extensively by cellular factors during the cell-cycle. In particular, the frequency of microtubule catastrophe (switch from growth to shrinkage) is increased significantly in MTs during mitosis relative to interphase, suggesting the existence of microtubule-destabilizing enzymes in the cell.[6,7] One such enzyme is XKCM1, identified in Xenopus eggs as a regulator

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Abbreviations used: MT, microtubule; SMT, microtubule made of subtilisin-cleaved tubulin; SMacro, macrotube made of subtilisin-cleaved tubulin; SRing, ring made of subtilisin-cleaved GDP-bound tubulin; AMPPNP, 5′-adenylylimidodiphosphate; GMPCPP, guanylyl (α,β)methylenediphosphonate.

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of microtubule dynamics during mitosis.[8] Its inhibition in extracts results in a fourfold reduction of the frequency of catastrophe, giving rise to long MTs that disrupt mitotic spindle assembly.[8]

XKCM1 is a member of the Kin I subfamily of kinesins. Unlike conventional kinesins, which convert the chemical energy of ATP hydrolysis into mechanical force for movement along MTs,[9] the Kin I kinesins exhibit no motile activity and function solely as microtubule-destabilizing enzymes.[10,11] XKCM1 directly targets microtubule ends and causes microtubule depolymerization.[10] This effect most likely occurs by promoting a destabilizing conformational change in the tubulin subunits. It has been proposed that ATP hydrolysis is not required for the destabilizing effect of XKCM1 on the microtubule, but rather is necessary for dissociation of XKCM1 from tubulin to permit recycling of the enzyme. XKIF2, and possibly other members of the Kin I subfamily, exhibit similar microtubule regulatory activity, suggesting that Kin I kinesins may be a major class of microtubule-destabilizers.[10–12] A detailed study of the mechanism of action of such regulators is necessary to further our understanding of regulation of cytoskeletal dynamics. In addition, microtubule dynamic regulators can be potential targets for antimitotic drugs that can be employed in therapeutic strategies.

To understand how XKCM1 functions, we have tested its ability to depolymerize different tubulin polymers (Figure 1) to test models of how it induces microtubule depolymerization. In zinc-induced sheets (Figure 1(b), left) and macrotubes (Figure 1(b), right) the individual protofilament conformation is similar to that in MTs, but the protofilaments are arranged in an anti-parallel fashion[13–15] with the lumenal and external faces of the protofilaments alternating. This topology has allowed us to discriminate between two possible general models of XKCM1-induced depolymerization; simultaneous binding of XKCM1 to a single or to two adjacent protofilaments. Closed ring polymers (Figure 1(c)), which resemble the depolymerized state of tubulin, are formed by GDP-tubulin in the presence of various divalent cations.[2,16,17] These structures are similar to the observed curls of protofilaments induced by XKCM1 depolymerization of GMPCPP-MTs,[10] and were exploited to increase our understanding of how XKCM1 binds to tubulin and MTs.

Our results show that XKCM1 is able to depolymerize tubulin zinc macrotubes. These results suggest that XKCM1 binds along a single protofilament during the depolymerization cycle. We find that the C terminus of tubulin is essential for the depolymerization activity of XKCM1, and that XKCM1 in the presence of a non-hydrolyzable ATP analog stabilized GDP-tubulin rings. Based on our results from depolymerization experiments as well as some structural modeling, we propose a model for how XKCM1 can induce depolymerization and what features of XKCM1 and the microtubule are essential for this biochemical activity.

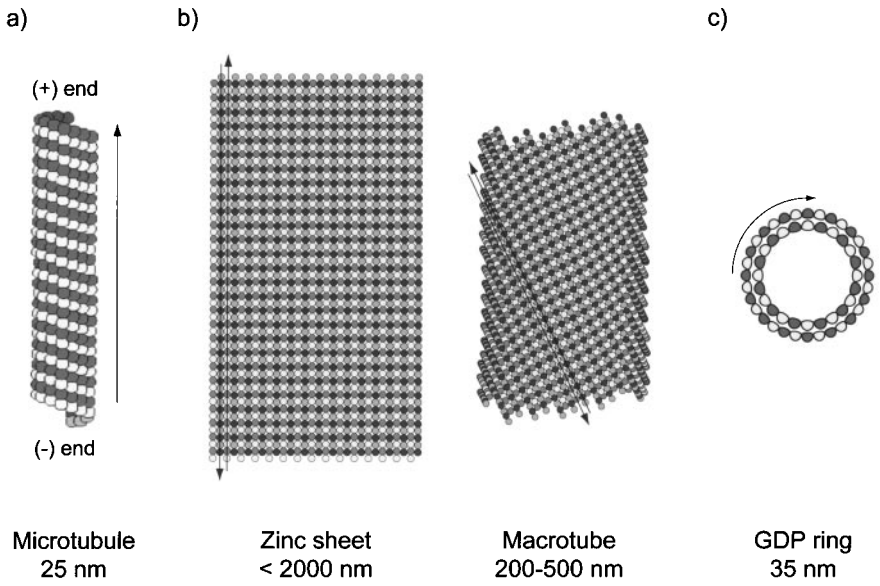

Figure 1. The arrangement of tubulin subunits in the different tubulin polymers. (a) Microtubule. The parallel arrangement of protofilaments and the polarity of the microtubule are indicated. (b) Zinc-induced sheets (left) and macrotubes (right) showing antiparallel protofilament arrangement. The different shades in adjacent protofilaments indicate the alternating in and out orientation of the subunits in consecutive protofilaments. (c) Curved protofilaments, characteristic of GDP-tubulin, form rings in the presence of stabilizing divalent cations. The polarity of one of the curved protofilaments is shown to indicate the protofilament topology relative to that found in a microtubule.

Results

Effect of XKCM1 on zinc-induced tubulin polymers *versus* MTs

We first tested the depolymerization activity of XKCM1 on MTs using a simple pelleting assay (Figure 2). When unpolymerized tubulin and XKCM1 are incubated together, XKCM1 was found in the soluble fraction along with unpolymerized tubulin (Figure 2, lanes 1 and 2). In the absence of XKCM1, but under conditions that promote microtubule assembly, the tubulin was now found in the pellet (Figure 2, lanes 3 and 4). When XKCM1 was added to a microtubule solution, XKCM1 quickly associated with MTs and started shifting tubulin from the pellet (assembled) to the soluble (depolymerized) fraction (Figure 2, lanes 5 and 6). After a 15 minute incubation, most of the tubulin, as well as the XKCM1 was in the soluble fraction (Figure 2, lanes 7 and 8).

In order two discriminate between our two simple models of XKCM1 action, one requiring simultaneous binding of XKCM1 to two adjacent protofilaments, *versus* one requiring binding to only one protofilament, we next tested the ability of XKCM1 to depolymerize zinc-induced tubulin polymers, which are formed by the antiparallel association of protofilaments. One concern was that these polymers are very sensitive both to free Zn^{2+} concentration and to pH. We first examined the stability of the tubulin zinc-induced sheets in XKCM1 buffer alone and saw rapid depolymerization (data not shown). Because the mixing of the zinc-containing buffer and the XKCM1 buffer resulted in an increase of the pH over that optimal for sheet formation, we decided to use zinc-induced macrotubes for future experiments (Figure 1(b), right). Macrotubes are an alternative zinc-induced tubulin polymer that form at a higher pH.[15] While still retaining the antiparallel organization of protofilaments, the sheet polymer curves and closes into macrotubes with a diameter of the order of 250 nm.

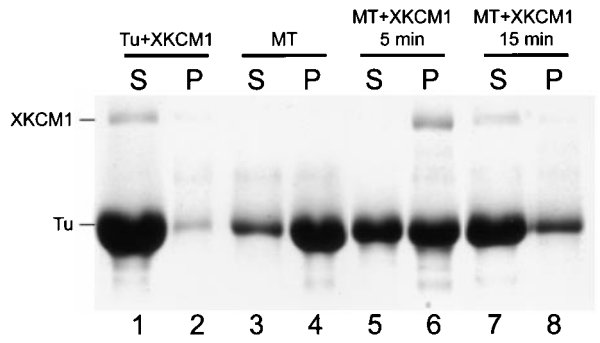

Figure 2. XKCM1 depolymerizes MTs. Lanes 1 and 2, tubulin was incubated without GTP or glycerol for 45 minutes at 37 °C: 0.6 μM XKCM1 was then added and the sample pelleted after 15 minutes at 37 °C. Lanes 3 and 4, MTs were grown in TB II at 37 °C for 45 minutes and then pelleted. Lanes 5 to 8, MTs were grown in TB II and 2.5 mM GTP for 45 minutes, at which point 0.6 μM XKCM1, 1.5 mM ATP was added and samples were pelleted at various times as indicated. XKCM1 is first seen in the pellet, but after 15 minutes is seen primarily in the supernatant.

We monitored the depolymerization process of tubulin macrotubes by light absorbance, electron microscopy, and by the pelleting assay used for MTs (Figure 3). Addition of XKCM1 buffer did not compromise macrotube stability, although a small (30 %) decrease in absorption was observed due to dilution (Figure 3(a), continuous line). EM observation showed no significant difference in macrotube amount or structure in samples in the presence or absence of XKCM1 buffer (data not shown). To test whether XKCM1 could depolymerize macrotubes, XKCM1 was added at a molar ratio of 1:80 XKCM1 to tubulin in the presence of 1.5 mM ATP. Under these conditions a 70 % decrease in absorbance was seen within three minutes (Figure 3(a), dotted line). These results were strengthened by EM analysis of the samples, which showed that there were no remaining macrotubes present, and only some protein aggregates could be observed (Figure 3(b)).

Sedimentation analysis of the samples showed similar results (Figure 3(c)). When the macrotube sample was pelleted before addition of XKCM1, most of the tubulin was present in the pellet (Figure 3(c), lanes 1 and 2). Three minutes after the addition of XKCM1, slightly more than half of the tubulin was depolymerized and present in the supernatant (Figure 3(c), lanes 3 and 4). After 15 minutes, over three-fourths of the tubulin is depolymerized (Figure 3(c), lanes 5 and 6). XKCM1 was present in the pellet and in the supernatant. This pellet was then resuspended in cold TB I buffer (see Materials and Methods) so that macrotubes, but not tubulin aggregates, would depolymerize. The solution was then centrifuged at 4 °C and both fractions assayed. About half of the tubulin is observed in this cold pellet, representing tubulin aggregates that exist in macrotube preparations, most likely due to the long incubation time (see Figure 3(b), left). All of the XKCM1 that was present in the warm pellet fractionated with the insoluble protein during the cold spin (Figure 3(c), lanes 7 and 8). These results indicate that XKCM1 binds irreversibly to tubulin aggregates or that part of the XKCM1 pool aggregates in the presence of Zn^{2+} and/or low pH. When the process was repeated with XKCM1 but in the absence of tubulin, XKCM1 did not form a pellet (data not shown), supporting the idea that there is irreversible binding of some XKCM1 to tubulin aggregates.

It has been proposed that ATP hydrolysis is required for recycling of the tubulin-XKCM1 complex.[10] In the absence of significant free ATP (total concentration of 9 μM), we observed a marked decrease in depolymerization of macrotubes by

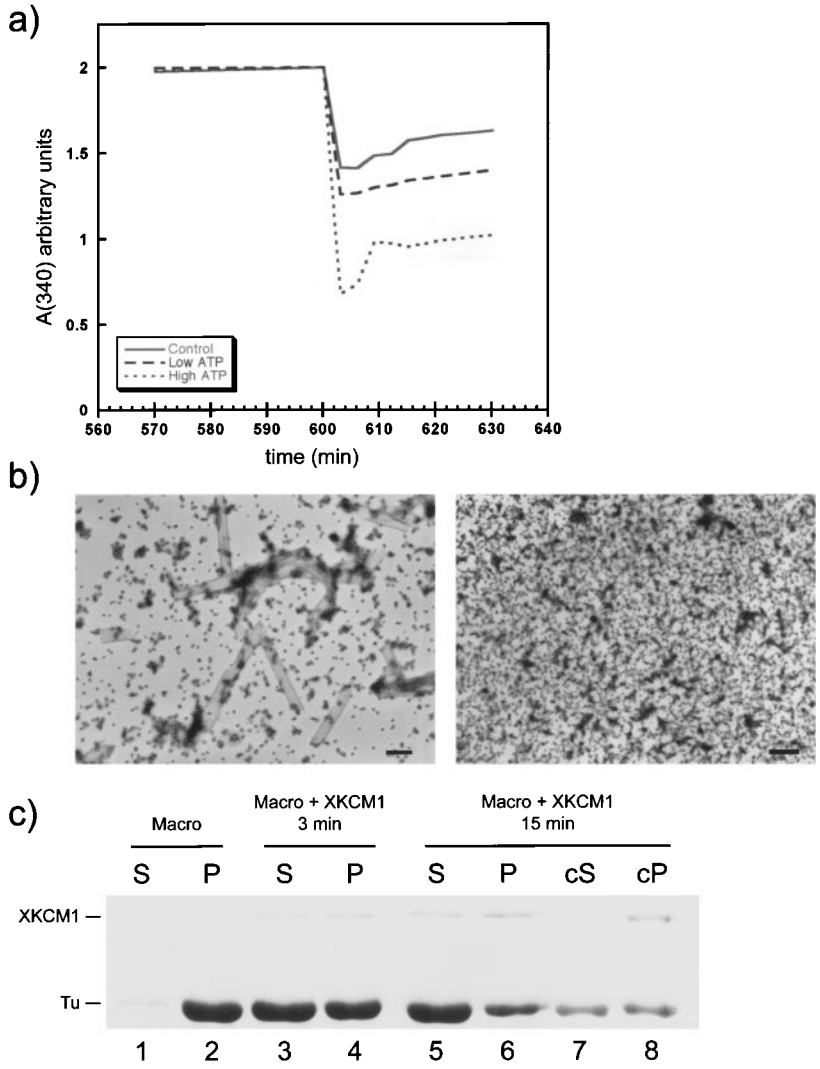

Figure 3. XKCM1 depolymerizes zinc-induced macrotubes. (a) Light absorbance traces for three macrotube samples as indicated in the text. The absorbance of the control tubulin macrotube solution falls 30 % from its original value following the addition of XKCM1 buffer due to sample dilution (continuous line). The absorbance of the macrotube solution decreases only 37 % from the original value following addition of XKCM1 under low ATP conditions (broken line). In the presence of 1.5 mM ATP, the absorbance drops 70 % from the original measurement (dotted line). (b) Electron micrographs of macrotube samples before (left) and after (right) addition of 0.6 μM XKCM1 in the presence of 1.5 mM ATP. The scale bars represent 500 nm. (c) Pelleting assay and SDS-PAGE analysis of XKCM1 depolymerization of macrotubes. Lanes 1 and 2, macrotubes were incubated for nine hours and then pelleted. Lanes 3 and 4, macrotubes were incubated in the presence 0.6 μM XKCM1 and 1.5 mM ATP for three minutes and then pelleted. Lanes 5 to 8, macrotubes were incubated with 0.6 μM XKCM1 and 1.5 mM ATP for 15 minutes and then pelleted. The pellet fraction was resuspended completely on ice, and then pelleted in a cold spin (cS, cold supernatant; cP, cold pellet).

XKCM1 (Figure 3(a), broken line). Some remaining macrotubes could be observed in these samples (not shown), further supporting the idea that the presence of excess ATP is required for multiple rounds of depolymerization by XKCM1.

Previous work has shown that motor proteins such as ncd (a microtubule minus end-directed motor protein) may disrupt the zinc sheet lattice when present at concentrations greater than 20 times that of tubulin.[18] This effect is most likely due to the fact that the binding site of kinesin and other kinesin-like motors on tubulin[19–21] overlaps partially with the region involved in lateral protofilament contacts in the zinc-induced polymers.[14,22] To show that XKCM1 has a unique macrotube-depolymerizing activity distinct from that of motile kinesins, we used conventional human kinesin as a control. We observed no depolymerization of the macrotubes by human kinesin for ratios up to 1:1 of kinesin to tubulin, 80 times that used in this study for XKCM1 (data not shown).

The C terminus of tubulin is required for XKCM1 activity

Because the C-terminal tail of tubulin has proven to be important for the processivity of several motile kinesins,[23,24] we wanted to test the effect of C-terminal tail removal on the depolymerizing action of XKCM1. Subtilisin-treated MTs (SMT) formed and pelleted similarly to MTs made with native tubulin (Figure 4, lanes 1 and 2) and look very similar in the electron microscope (data not shown). When SMTs were incubated with XKCM1 they did not depolymerize, even at XKCM1 to tubulin ratios as high as 1:20 (Figure 4, lanes 3-6). Electron micrographs of these samples showed abundant MTs of normal appearance (data not shown). Although depolymerization did not occur, XKCM1 appeared in the pelleted fraction, indicating that it does bind to subtilisin-treated MTs. To test whether the XKCM1 present in the subtilisin-cleaved microtubule pellet was not simply bound to aggregated tubulin, the pellet was resuspended

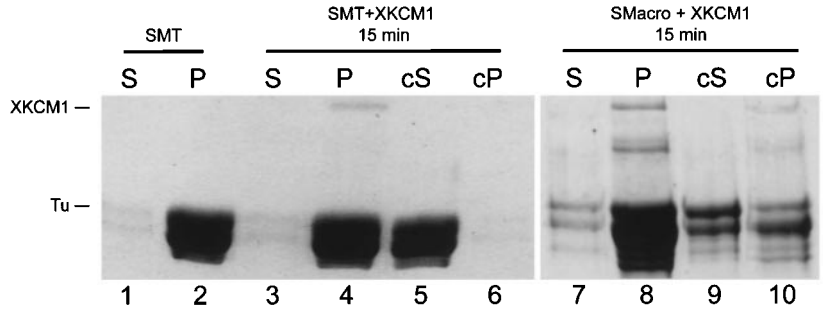

Figure 4. XKCM1 is unable to depolymerize subtilisin-cleaved MTs or macrotubes. Lanes 1 and 2, subtilisin-digested tubulin (STu) was polymerized into MTs (SMT) in CB II and pelleted after 45 minutes. Lanes 3 to 6, subtilisin-digested MTs were incubated with 0.6 μM XKCM1 and pelleted after 15 minutes. The pellet (P) was resuspended thoroughly in cold TB I and cold-pelleted (cS and cP) for analysis. Most of the STu is seen in the supernatant of the cold spin along with the majority of the XKCM1, indicating that the XKCM1 is bound mostly to polymerized SMTs. Lanes 7 to 10, macrotubes made from STu were incubated with 0.6 μM XKCM1 for 15 minutes and pelleted. The pellet fraction was resuspended on ice in TB I and cold fractionated. XKCM1 seems to form insoluble aggregates with tubulin.

in cold TB I buffer so that MTs, but not tubulin aggregates, would depolymerize. The solution was then centrifuged at 4 °C, and both fractions were analyzed. XKCM1 fractionated mostly as soluble protein (Figure 4, lanes 5 and 6). This result suggests strongly that XKCM1 interacts with polymerized, subtilisin-cleaved tubulin but is unable to depolymerize these MTs.

When macrotubes made from subtilisin-treated tubulin (SMacro) were made, they also were not depolymerized by XKCM1 (Figure 4, lanes 7 and 8). Again, all of the XKCM1 appears in the pelleted fraction. When the SMacro pellet was cold resuspended, only half of the tubulin was present in the cold soluble fraction (Figure 4, lanes 9 and 10), indicating that a significant part of the subtilisin-treated tubulin aggregates during the macrotube incubation. As with the native tubulin, XKCM1 came down with this pellet. The absence of XKCM1 in the soluble cold fraction (Figure 4, lane 10) indicates that XKCM1 does not bind to macrotubes made of tubulin lacking the C terminus.

Effect of XKCM1 on GDP-tubulin rings

We were interested in finding out whether XKCM1 can bind to and depolymerize GDP-tubulin rings, which are made of curved protofilaments similar to microtubule depolymerization fragments (Figure 1(c)). We had two problems that needed to be overcome. First, the stabilization of the GDP conformation of tubulin into rings is non-trivial. This obstacle was solved by removal of the C-terminal tail of the protein, whereupon stability of this polymer form was enhanced greatly. Although our experiments with MTs and macrotubes using subtilisin cleaved-tubulin clearly showed that the C terminus is required in these polymers for XKCM1-induced depolymerization, we decided to test the subtilisin-cleaved form of the rings (SRings) first. This allowed us to test if the same trend existed for rings, and to check if binding of XKCM1 to this polymer form occurs, irrespective of depolymerization activity. The second problem had to do with the fact that high salt is required for the stability of XKCM1. XKCM1 is eluted during purification and stored at KCl concentrations of approximately 300 mM. This high KCl concentration resulted in the rapid disappearance of the SRings and the formation of large tubulin precipitates (not shown). However, XKCM1 is functional at KCl concentrations down to approximately 120 mM. At this concentration of KCl, amorphous tubulin aggregates still formed to a certain degree, but numerous SRings were still present. The proportion of SRings to aggregates was reduced by decreasing the tubulin concentration threefold to 1.5 mg/ml. The presence of Mn^{2+} concentrations between 8 and 12 mM was also helpful in stabilizing the SRings upon dilution of tubulin to 0.8 mg/ml. Dilution of tubulin below 0.7 mg/ml usually led to large-scale depolymerization of the rings. Thus, we concluded that the use of reduced KCl and tubulin concentrations in the presence of moderate Mn^{2+} concentrations resulted in GDP-tubulin SRings that were stable enough to proceed with functional studies of XKCM1.

High-speed centrifugation and electron microscopy were used to test the extent to which XKCM1 was able to depolymerize SRings. As a control, we observed that SRings were not depolymerized by XKCM1 buffer (Figure 5(a), lanes 1 and 2). When the SRings were incubated with XKCM1, the enzyme was unable to depolymerize them, either in the presence of low (<10 μM) or high (1.5 mM) ATP concentration, even at XKCM1 to tubulin ratios as high as 1:6 (Figure 5(a), lanes 3-6). Electron micrographs of these samples (Figure 5(b)) showed SRings indistinguishable from those obtained in the absence of XKCM1 (not shown). After high-speed centrifugation, XKCM1 was found mostly in the pellet. As XKCM1 by itself does not precipitate in the same buffer conditions (data not shown), our results suggest that XKCM1 binds to the SRings.

As expected, XKCM1 in the presence of the non-hydrolyzable ATP analog AMPPNP also did not depolymerize S rings (Figure 5(a), lanes 7 and 8). Instead, AMPPNP-XKCM1 generally stabilized closed SRings, reducing both the amount of ring

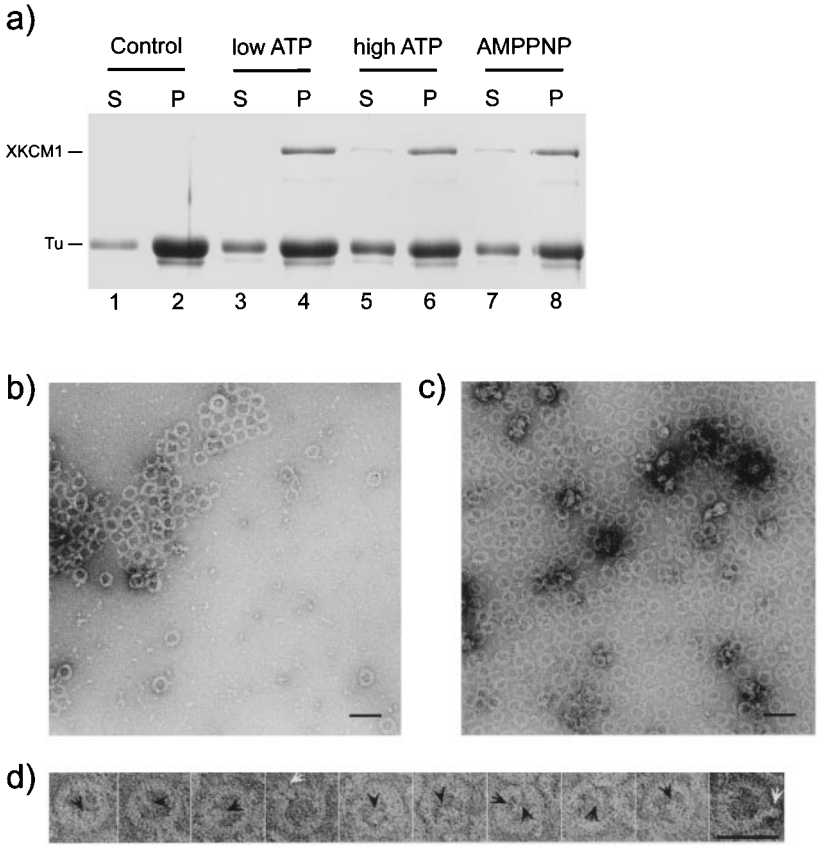

Figure 5. XKCM1 does not depolymerize GDP-tubulin rings. (a) Pelleting assay and SDS-PAGE analysis showing that GDP-tubulin SRings are resistant to XKCM1 depolymerization. Lanes 1 and 2, control ring samples were pelleted for 12 minutes at 100,000 rpm and analysed by SDS-PAGE. Lanes 3 and 4, ring samples incubated with 0.6 μM XKCM1, 9 μM ATP for ten minutes and then pelleted. Lanes 5 and 6, ring samples incubated with 0.6 μM XKCM1 and 1.5 mM ATP for ten minutes and then pelleted. Lanes 7 and 8, GDP-tubulin SRings were incubated with 0.6 μM XKCM1, 5 mM AMPPNP and then pelleted. (b) Electron micrograph of GDP-tubulin SRings ten minutes after the addition of 0.6 μM XKCM1, 1.5 mM ATP. GDP-tubulin rings are present and numerous. (c) Electron micrograph showing GDP-tubulin rings in the presence of 0.6 μM XKCM1 and 5 mM AMPPNP. The quantity of closed, well-segregated GDP-tubulin rings is increased greatly after addition of XKCM1 in the presence of the non-hydrolyzable ATP analog. The scale bars represent 100 nm. (d) Selected rings from (c) showing attached, extra densities that could correspond to bound XKCM1. Black arrows indicate densities inside the ring, the white arrows indicate densities outside the ring. The scale bar represents 50 nm.

fragments and the aggregation of rings often seen in other conditions (Figure 5(c)). This stabilization is directly attributable to XKCM1, as SRing samples looked indistinguishable before and after addition of AMPPNP by itself (data not shown). Even in conditions that promote paracrystalline association of rings (Mn^{2+} concentrations lower than 6 mM or high tubulin concentrations), a most dramatic reduction in the number of paracrystals was obtained in the presence of AMPPNP-XKCM1. To control against a possible general effect of kinesins, incubation with excess conventional human kinesin had no effect on the stability of these SRings or the formation of ring aggregates, regardless of the nucleotide used (data not shown).

In order to see if the lack of depolymerization activity on the GDP-rings was due to the absence of the tubulin C-terminal tail of tubulin, we repeated the depolymerization assay with rings formed from native tubulin protein. To generate GDP-tubulin rings with an intact tubulin C terminus, the Mn^{2+} concentration had to be increased to 40 mM. Rings made from this native tubulin were also not depolymerized by XKCM1 in the presence of ATP when assayed by a pelleting assay and by electron microscopy (data not shown). However, it was possible that the presence of 40 mM Mn^{2+} rendered XKCM1 unable to depolymerize any tubulin polymer. In agreement with this possibility, taxol-stabilized MTs, which are depolymerized readily by XKCM1[10] and resistant to the high Mn^{2+} concentrations (data not shown), were also not depolymerized by the enzyme in the presence of 40 mM Mn^{2+}. Thus, it appears that the buffer conditions required for the formation of stable GDP- rings with native tubulin are incompatible with XKCM1 activity.

Discussion

XKCM1 acts on a single protofilament

XKCM1, a member the Kin I subfamily of kinesins, exhibits no motor activity but rather functions as a microtubule-depolymerizing enzyme. The binding of XKCM1 to MTs *in vitro* is sufficient to induce depolymerization, while ATP hydrolysis is most likely involved in recycling the enzyme from a complex with tubulin.[10] In our study, we tested two general models for how XKCM1 binding could cause a depolymerizing conformational change in MTs: whether XKCM1 dimers bind two adjacent protofilaments and force them apart (Figure 6, model 1), or whether XKCM1 binds along a single protofilament and disrupts its nor-

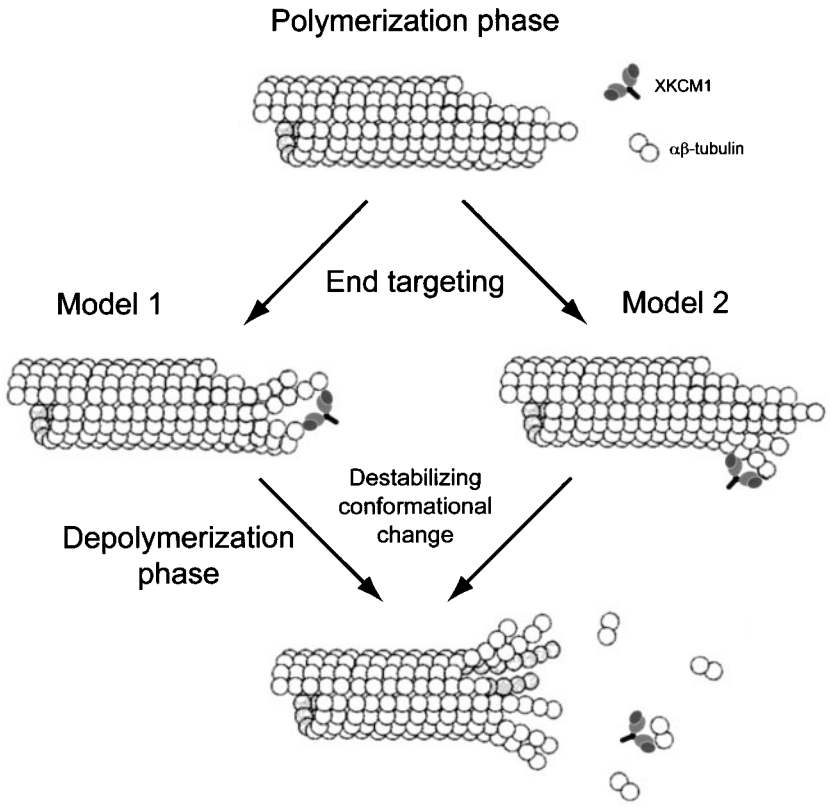

Figure 6. Alternative, general models of XKCM1 depolymerization. Proposed models of XKCM1 activity. Model 1, XKCM1 interacts with two adjacent protofilaments to disrupt the microtubular lattice. Model 2, XKCM1 interacts with just one protofilament to disrupt or alter lateral interdimer contacts.

mal longitudinal or lateral interactions (Figure 6, model 2). In order to distinguish between these two models, we examined the ability of XKCM1 to depolymerize tubulin polymers made of antiparallel protofilaments. These polymers cannot provide physiological surfaces for simultaneous contact of both XKCM1 heads within the dimer as required for model 1. We observe that zinc-induced macrotubes are depolymerized by XKCM1 at concentrations similar to those used for the disruption of MTs *in vitro*.[10] This result rules out model 1 and leads us to propose a general model for XKCM1 activity in which the XKCM1 dimer binds along a single microtubule protofilament (model 2) and causes a conformational change in the protofilament that leads to depolymerization. This change in conformation could be due to simultaneous binding of the two heads of the XKCM1 dimer to two consecutive sites on the microtubule, causing a bending angle in the protofilament by decreasing the distance between the two tubulin units. Alternatively, it may mean that the binding of a single head of XKCM1 is sufficient for depolymerization. We have recently found that XKCM1 constructs lacking an intact dimerization domain also exhibit depolymerization activity, supporting this latter hypothesis (C.W., unpublished results). Similarly, recent results with monomeric versions of MCAK, the hamster XKMC1 homologue, show that these proteins also maintain their depolymerization activity.[11] The authors conclude that depolymerization does not require coupling the activity of two heads. Our experiments with tubulin zinc macrotubes expand on this hypothesis by showing that the interaction is to a single protofilament. Therefore, depolymerization clearly is not induced by the two monomers spanning two protofilaments, but it also does not occur by a single monomer binding simultaneously to two adjacent protofilaments (across an interprotofilament interface). While this is an obvious conclusion from our experiments, the question still remains about how the binding of a single XKCM1 catalytic head to a single protofilament has such a disruptive effect on microtubule stability. Furthermore, we still do not know how XKCM1 binds selectively to both microtubule ends. It may be able to recognize the structure of the GTP cap, or perhaps its binding site may require surfaces that are exposed only at microtubule ends. These surfaces cannot be those involved in longitudinal contacts between tubulin dimers, as such surfaces are completely different at the plus and minus ends. We favor the idea that the XKCM1 binding surface on tubulin overlaps partially with that involved in lateral contacts between tubulin protofilaments. A number of such surfaces should always be exposed at both microtubule ends.

It is important to mention that conventional kinesin cannot walk on zinc-induced polymers[25] and that the binding site of both kinesin and ncd overlap partially with the protofilament interface

in these polymers.[19,20,22] As both zinc-induced sheets and the macrotubes used in this study have basically the same protofilament arrangement,[15] it is reasonable to assume that the same limitations in binding and motility apply to these types of polymers. Thus, it is particularly interesting that XKCM1 is able to bind to and depolymerize macrotubes, with an efficiency similar to that seen on MTs. This is another example of how the mechanism used by XKCM1 to induce microtubule depolymerization is distinct from that used by motile kinesins for conventional motility.

XKCM1 requires the tubulin C terminus for depolymerization

The C terminus of tubulin is a highly acidic and presumably disordered region of the tubulin molecule.[26] In the electron crystallographic map, this region had very weak or non-existent density, in agreement with a lack of structure.[14] The position of the last residues in the atomic model within the high-resolution docking of the structure into the microtubule envelope[27] indicated that this disordered tail is exposed on the microtubule surface, in agreement with its known interaction with MAPs[28,29] and its accessibility to proteases. *In vitro* cleavage of this acidic tail by subtilisin reduces the critical concentration for tubulin assembly and facilitates the formation of a variety of polymer forms.[26,30,31] A general model proposes that removal of the tail overcomes an electrostatic repulsion between the tubulin subunits. Moreover, it has been shown that the C terminus of tubulin is important for the motility of several kinesins. The monomeric kinesin KIF1A interacts with the C terminus of tubulin through its positively charged K-loop to achieve processivity.[21,23] Furthermore, it has been shown that conventional kinesin interacts with the negatively charged tail to increase its processivity several-fold.[24] These findings motivated us to test for the possible effect of the C terminus of tubulin in XKCM1-induced depolymerization.

Our experiments show that XKMC1 requires the C terminus of tubulin for its depolymerizing activity, both on MTs and on zinc-induced macrotubes. However, our data also suggest that the C terminus is not required for the binding of XKCM1 to MTs. Given the overall conservation of the catalytic domain of all kinesins, which includes the microtubule-binding site identified for conventional kinesin and ncd, it is not surprising that XKCM1, like other kinesins tested, still binds, although most likely with a reduced affinity, to the microtubule surface in the absence of the C terminus of tubulin. Interestingly, when this C-terminal tail is not present in zinc-induced macrotubes, where the binding site for conventional kinesin is partially occluded by lateral contacts, XKCM1 is unable to depolymerize these polymers, and unable to bind to them. Finally, it is worthwhile to notice that the high Mn^{2+} concentrations that help in the formation of the native tubulin rings, most likely by screening the negative charge of the C terminus in tubulin, render XKCM1 incapable of depolymerization, perhaps also by this screening effect. Alternatively the high Mn^{2+} concentration may render the enzyme inactive, independently of its binding to tubulin. An ATPase assay of XKCM1 at these Mn^{2+} concentrations would be required to discriminate between these two possibilities. We believe it is reasonable to speculate that XKCM1 has an altered binding affinity for subtilisin-cleaved tubulin and will investigate this point further.

XKCM1 stabilizes the curved GDP conformation of tubulin

Upon depolymerization, MTs form curved oligomers that can close into rings.[3,5] Free GDP-tubulin subunits can be stabilized into curved oligomers and rings by divalent cations.[2] During XKCM1 depolymerization of GMPCPP-MTs, long protofilament curls are seen at the depolymerizing ends,[10] implying that XKCM1 is disrupting lateral contacts and/or inducing a curved conformation of the protofilament, but without necessarily eliminating longitudinal interactions. These results lead us to think that XKCM1 could bind to GDP-tubulin rings without disturbing their structure. Our present results cannot fully confirm this prediction, as our ring-stabilizing conditions, subtilisin cleavage of the tubulin C terminus or high Mn^{2+} concentration, are incompatible with the depolymerizing activity of the enzyme even when tested on MTs. However, under these conditions XKCM1 seems to bind to tubulin rings as judged by pelleting assays. Most of the rings are closed structures without accessible longitudinal interfaces. As expected from the binding of XKCM1 to both microtubule ends, which have very different longitudinal interfaces exposed, this result confirms that binding does not occur at a tubulin surface involved in longitudinal contacts. Rather, we believe that the extended binding site of XKCM1 covers that of conventional kinesin on the microtubule outer surface, and part of a lateral interface that is accessible in GDP-tubulin rings as well as both ends of the microtubule. Furthermore, in the presence of AMPPNP, XKCM1 causes certain stabilization of closed, individual rings, by reducing the amount of ring fragments and practically eliminating their aggregation into higher-order structures. Our electron microscopy images do not show the interaction of XKCM1 with rings very clearly, even when using a 1:6 concentration of XKCM1 to tubulin. A significant number of the rings seem to have extra density on the inside of the inner ring (Figure 6(c), black arrows), but in some cases extra density is observed outside the ring (Figure 6(c), white arrows) Thus, it is possible that the extra density could correspond to unpolymerized tubulin or unbound XKCM1 that, by random chance, falls close to the ring structure. The use of gold-

antibody labeling may be required to confirm the binding suggested by these images.

Structural elements in XKCM1 implicated in the depolymerization activity

Initial studies of XKCM1 suggested that no ATP hydrolysis is required for a single round of depolymerization.[10] Thus, it seems reasonable to assume that energy for this event comes directly from the energy of binding of XKCM1 to the microtubule. In the absence of the C terminus of tubulin, the energy of binding may be reduced so that no significant conformational change is produced in the tubulin subunits. Our present hypothesis is that the acidic C terminus of tubulin interacts directly with XKCM1, most likely involving basic residues in the enzyme that may be unique to the Kin I family. This idea led us to analyze the known sequences of members of the Kin I family and to compare them with conventional kinesin in the context of the crystal structure of the motor domain and its interaction with MTs (Figure 7).

The Kin I family is characterized by the position of the catalytic core in an internal region of the primary sequence, preceded by a globular amino-terminal domain that is involved in targeting XKCM1 to the kinetochore (C.W., unpublished results). Deletion of most of this N-terminal globular region, however, yields XKCM1 that retains its depolymerizing activity (C.W., unpublished results). Similar results have been obtained with MCAK.[11] Still, a few residues N-terminal to the catalytic domain are required for the action of Kin I proteins. There is no atomic structure of this region, which contains about 30 amino acid residues on the N-terminal end of the catalytic domain, but secondary structure prediction for a peptide containing the 50 N-terminal amino acid residues to the catalytic domain show a very large likelihood of a 30 residue, very hydrophilic α helix, followed by a shorter, amphipathic helix (data not shown). The N terminus of the catalytic domain from which this segment should protrude, is near the surface that contacts the microtubule, not far from a longitudinal interface between tubulin subunits, and level with the last C-terminal residues of the tubulin structure that lead to the acidic tail (Figure 7(b) and (c), asterisk, model based on the binding of conventional kinesin and KIF1A to the microtubule[21]). The answer to the unresolved question of how XKCM1 targets microtubule ends may indeed involve this region and its possible interaction with lateral surfaces on the protofilament distinctive from those on the outside surface involved in binding to the conserved catalytic domain.

On the other hand, in addition to the amino-terminal domain, there are several changes within the catalytic core that are unique to Kin I kinesins and are thus possible candidates to be involved in microtubule interaction and depolymerization. The most likely candidate sequence is an inserted loop (labeled Tu C-term loop in Figure 7, blue) near the beginning of the XKCM1 catalytic domain, and thus close to the required residues in the N-terminal domain. The loop is positively charged and lies near the location of the negatively charged tubulin C terminus.[21,32] This loop is conserved throughout the Kin I family but is not present in other kinesins. In addition, Kin I kinesins have a cluster of conserved amino acid changes from hydrophilic to basic (Figure 7 red, Lys/Arg loop). Their location partially overlapping the MT1 binding region (loop 8a in KIF1A) might affect the affinity of XKCM1 to tubulin, or even contribute to the destabilization of the microtubule lattice. A third conserved difference between Kin I kinesins and conventional kinesin is a deletion in the loop between the two β strands that comprise the tip of the kinesin ''arrowhead'', but this difference is located far from any of the microtubule binding sites (Figure 7, magenta).

Interestingly, the HIV-1 Rev protein has recently been shown to depolymerize MTs and induce the formation of tubulin rings.[33] Sequence analysis of part of this protein shows a high level of homology to the microtubule-binding region of the catalytic domain of XKCM1 and XKIF2 (Figure 7, cyan). It is important to note that this part of the kinesin lies at the interface between the two tubulin monomers, and could possibly be involved in inducing a kink into the protofilament structure. This further suggests the involvement of the catalytic domain of XKCM1 in the microtubule depolymerization process.

Conclusions

The present study strongly supports a model in which XKCM1 binding to tubulin is restricted to a single protofilament. While the enzyme can still bind to microtubules that lack the C-terminal tail of tubulin, this acidic segment is required for the XKCM1 destabilizing action. Conserved regions within the catalytic core unique to Kin I proteins that are rich in basic residues are located on the surface facing the microtubule and in positions where they are likely to interact with the acidic C-terminal of tubulin. A mutational analysis of these regions will be important to determine how they affect the depolymerization ability of XKCM1.

Materials and Methods

Tubulin and XKCM1 purification

Porcine brain tubulin was purified by several warm/cold cycling steps as described.[34] Aliquots of tubulin (18 mg/ml) in tubulin buffer I (TB I: 80 mM Pipes, 1 mM EGTA, 1 mM $MgCl_2$) were frozen and stored at −80 °C. MAP-free bovine tubulin in TB I at 10 mg/ml from Cytoskeleton, Inc. (Boulder, CO) was used in some cases. XKCM1 was purified from an Sf-9/baculovirus expression system through a combination of ion-exchange and gel-filtration chromatography as

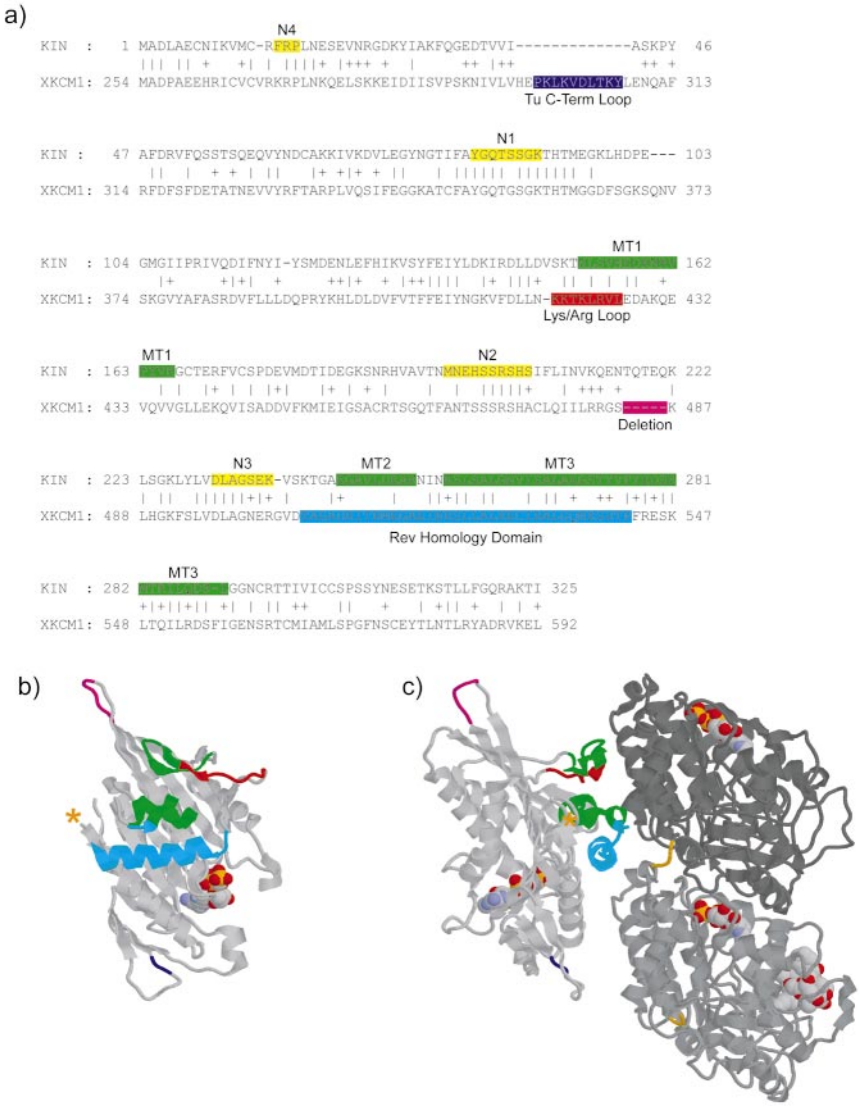

Figure 7. Sequence analysis of the XKCM1 catalytic domain and localization of regions unique to Kin I proteins. (a) Homology-based sequence alignment of the conserved catalytic domain of conventional kinesin (accession code 1BG2) and the central catalytic domain of XKCM1 (residues 254-592) show several important differences between the two proteins. The nucleotide-binding pocket (yellow) and MT-binding (MTx) regions (green) (based Kikkawa *et al*.[21]) are highlighted on the top line. Important differences between conventional kinesin and XKCM1 are marked on the bottom line (Tu C-term loop, blue; Lys/Arg loop, red; deletion, magenta). The region homologous to part of the HIV-1 Rev protein is highlighted in cyan. (b) View of the catalytic domain of KIF1A[37] as seen from the microtubule (the MT axis runs vertically with the plus end towards the top). The structural areas discussed in the text are color-coded as in (a). (c) Side-view of the motor domain (rotated 90° from (b)) interacting with an αβ-tubulin dimer. The last three residues in the atomic model of α and β-tubulin are highlighted in orange. The first residue of the catalytic domain of XKCM1 is marked by an asterisk. We have used the relative positions of the motor domain and tubulin from the docking experiments of KIF1A bound to MTs.[21] The relative positions of α and β-tubulin are arbitrary, as the reconstruction used for the docking did not have enough resolution to distinguish between the two tubulin monomers. For both (b) and (c), the nucleotides in tubulin and KIF1A, as well as the taxol molecule in β-tubulin, are shown in CPK form. This Figure was generated with RasMol.

described.[10] XKCM1 fractions eluted from the Mono S column were supplemented with 10% (w/v) sucrose, flash-frozen in liquid nitrogen and stored at −80°C.

Tubulin zinc sheet and macrotube formation

Tubulin aliquots were thawed on ice and added to zinc sheet/macrotube incubation buffer (IB: 80 mM Mes, 200 mM NaCl, 4.5 mM GTP, 50 μg/ml of pepstatin, and 1 mM $MgSO_4$, pH 5.7 for zinc sheets, pH 6.0-6.2 for macrotubes) to a final tubulin concentration of 5 mg/ml. $ZnSO_4$ was added to a final concentration of 0.5 mM and 0.6 mM for zinc sheets and macrotubes, respectively.[15,25] The high NaCl concentration and the presence of the protease inhibitor pepstatin allowed for better preservation of the polymers for prolonged incubation periods.[15] Sheet and macrotube samples were incubated at 32°C. Sheets were assayed after 24 hours to allow for maximum growth, while macrotubes were analyzed after ten hours to maximize the amount of macrotubes *versus* tubulin aggregates.

XKCM1 macrotube depolymerization assays

After polymer formation, XKCM1 protein solution or control XKCM1 buffer (XB: typically 100 mM KCl, 2 mM MgATP, 80 mM Pipes (pH 6.8), 1 mM EGTA, 1 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 10% (v/v) glycerol) were added to polymerized sheets or macrotubes to the concentrations indicated in the text. Polymer depolymerization was followed either by electron microscopy (EM), a pelleting assay, and/or by light absorbance.

At different time-points in the polymerization/depolymerization reaction, 5 μl samples were taken for EM analysis. Samples were adsorbed onto 400 mesh, carbon-coated copper grids. The sample drop was blotted, rinsed with deionized water, stained with 2% (w/v) uranyl acetate, and blotted to dryness. The grids were

observed using a JEOL 1200EX transmission electron microscope operated at 80 kV. Magnifications used ranged from 10,000× to 30,000×.

Typically, 15 minutes after XKCM1 addition, macrotubes were pelleted for 12 minutes at 13,200 rpm in an Eppendorf F45-36-8 rotor at room temperature. The supernatant was removed and the pellet was resuspended in cold TB I. The samples were analyzed by SDS-PAGE alone. In certain cases, indicated in the text, the first pellet was resuspended on ice and subjected to a cold spin at 12,500 rpm in a Napco 2028R refrigerated centrifuge to separate soluble proteins from insoluble aggregates generated during the incubation. The cold-spin pellet was again resuspended and analyzed alongside the cold-spin supernatant by SDS-PAGE. Light absorbance at 340 nm was used to observe the changes in the polymerization state of tubulin over time. A water-bath was used to keep the sample at the desired temperature (32 °C for sheets and macrotubes) during the time-course of the observations. Readings were taken at one minute intervals for incubations of 60-100 minutes and at three minute intervals for prolonged incubations.

Subtilisin cleavage of tubulin

In order to obtain consistent cleavage for experiments requiring subtilisin cleaved tubulin (STu), we always used tubulin assembled into MTs as the subtilisin substrate.[35] Tubulin aliquots were thawed on ice and diluted to 6 mg/ml in TB II (TB I plus 10 % glycerol) and 2.5 mM GTP. The mixture was incubated at 37 °C for 30-45 minutes to form MTs. The microtubule sample was incubated with 20 μg/ml of subtilisin at 30 °C. Subtilisin cleavage was stopped after 12 minutes by the addition of 2 mM PMSF (in DMSO), and MTs were pelleted at 13,200 rpm for 12 minutes (Eppendorf F45-36-8 rotor). The pellet was washed once with TB I and resuspended to the appropriate tubulin concentration in either TB II for MTs (SMT) and macrotubes (SMacro) or low Mn^{2+} ring buffer (see the next section) for GDP-tubulin rings (SRings).

GDP-tubulin ring studies

As for many other tubulin polymer types,[26,31] formation of rings by GDP-tubulin is enhanced by subtilisin cleavage.[36] Subtilisin-cleaved tubulin was prepared as described above and resuspended in cold ring buffer (TB I plus 2 mM GDP, 12 mM $MnSO_4$) to a final tubulin concentration of 1 mg/ml. Rings were formed by incubating at 37 °C for 30 minutes. In some cases, GDP-tubulin rings were made from tubulin that was not previously digested with subtilisin. In these instances, the thawed tubulin aliquots were diluted directly to 1 mg/ml in ring buffer containing 40 mM $MnSO_4$. The extra Mn^{2+} is required for ring stabilization in the presence of the acidic C-terminal tail of tubulin.

Prior to XKCM1 addition, ring aliquots were centrifuged in a Beckman TLA 100 rotor at 35,000 rpm at 37 °C for 12 minutes to remove large aggregates: 70 mM KCl and 1 mM DTT were added to the supernatant in order to approximate the salt content of the ring buffer to that of the final XKCM1 buffer. ATP or AMPPNP were also added to a final concentration of 1.5 mM or 5 mM, respectively, as indicated. After ten minutes incubation with XKCM1, GDP-tubulin rings were pelleted for 12 minutes in a TLA 100 rotor at 100,000 rpm at 37 °C. The supernatant was removed, and then the pellet was washed and resuspended in an equal volume of TB I. Equal amounts of the pellet and supernatant were analyzed by SDS-PAGE.

EM grids were prepared at different points as for the zinc-induced polymers and observed using a Philips Tecnai 12 transmission electron microscope operating at 120 kV. Magnifications used ranged from 10,000× to 49,000×.

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