XMAP215 polymerase activity is built by combining multiple tubulin-binding TOG domains and a basic lattice-binding region

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Members of the XMAP215/Dis1 family are characterized by a varying number of TOG domains at their N termini (Fig. 1). Based on mutants in TOG domains that interfere with tubulin binding (15) and protein activity (16–20), it has been proposed that TOG binding to tubulin is required for its catalytic activity (21); however, there is no proof for this idea. It is also not known how the various properties of XMAP215—association with the tubulin dimer, binding to the microtubule lattice and plus end, diffusion along the lattice—depend on the TOG domains. We have therefore sought to determine how the TOG domains, and possibly other domains, contribute to microtubule polymerase activity.

**Results**

**TOG Domains are Required to Bind Free Tubulin and for Microtubule Polymerization.** Two key loops in TOG3 of Zyg9, the XMAP215/Dis1 homolog in *Caenorhabditis elegans*, were previously identified as being important for interaction with free tubulin (15). We mutated two conserved residues in the corresponding conserved loops of all five TOG domains of XMAP215 to determine their contribution to microtubule growth promotion (Fig. 1). This TOG1-5AA mutant did not promote growth under any conditions (Fig. 2 A–C). We assayed for growth above 5 μM tubulin because no growth is seen at or below this concentration in the absence of any growth-promoting factor due to the high frequency of catastrophes at low tubulin concentrations (14). We then assayed this mutant for the other properties characteristic of the wild-type protein: free tubulin binding, microtubule binding, microtubule lattice diffusion, and tip tracking. The mutant’s affinity for free tubulin was severely reduced, as shown by size exclusion chromatography (Fig. 2 B and C).

**TOGs 1 and 2 are Critical for Polymerase Activity.** These data demonstrate that the TOG domains play an essential function in mediating XMAP215’s ability to bind free tubulin and in promoting microtubule growth; however, they do not allow us to assess the contribution of individual TOG domains. To address this question, we generated a series of constructs in which TOG domains were sequentially deleted from the corresponding domains of the wild-type XMAP215 protein. Each of these mutants had a wild-type polymerase activity and a wild-type ability to bind free tubulin and in promoting microtubule growth (Fig. 1). This polymerase activity and its dependency on the concentration of its substrate, tubulin. However, how the various domains of the XMAP215 protein contribute to these activities is not known.

**Discussion**

**The Contribution of Individual TOG Domains to Polymerase Activity.** We used site-directed mutagenesis to determine the relative contributions of the individual TOG domains to microtubule growth. To address this question, we generated a series of constructs in which TOG domains were sequentially deleted from the corresponding domains of the wild-type XMAP215 protein. Each of these mutants had a wild-type polymerase activity and a wild-type ability to bind free tubulin and in promoting microtubule growth (Fig. 1). This polymerase activity and its dependency on the concentration of its substrate, tubulin. However, how the various domains of the XMAP215 protein contribute to these activities is not known.

**Additional Experimental Details.** We assayed for growth above 5 μM tubulin because no growth is seen at or below this concentration in the absence of any growth-promoting factor due to the high frequency of catastrophes at low tubulin concentrations (14). We then assayed this mutant for the other properties characteristic of the wild-type protein: free tubulin binding, microtubule binding, microtubule lattice diffusion, and tip tracking. The mutant’s affinity for free tubulin was severely reduced, as shown by size exclusion chromatography (Fig. 2 B and C).

**Author Contributions.** P.O.W., J.H.S., A.R., and S.B. performed research; P.D.W., I.H.S., M.Z., G.J.B., A.A.H., and J.H. analyzed data; and P.O.W., J.H.S., M.Z., A.A.H., and J.H. wrote the paper.

**Conflict of Interest.** The authors declare no conflict of interest.

**Funding.** This article is a *PNAS* Direct Submission. Freely available online through the *PNAS* open access option.

**Supplemental Material.** This article contains supporting information online at www.pnas.org (lookup/suppl/doi:10.1073/pnas.1016498108/-/DCSupplemental).
were individually or pair-wise mutated. We tested the ability of these combinations of TOG mutants to promote microtubule growth using two different assays. First, we determined the activities of the various mutants at a fixed tubulin concentration of 5 μM. No growth was seen from microtubule seeds with 5 μM tubulin alone (as noted above). However, when full length XMAP215 was added, we saw a dose dependent increase in growth rate that reached a maximum at approximately 200 nM protein. All functional point mutants reached maximum growth at this concentration but with significantly different maximum growth rates (Fig. 3A). And second, we measured the microtubule growth rate over a range of tubulin concentrations at a fixed XMAP215 concentration of 200 nM. The growth rates increased linearly with tubulin concentration for all mutants; however, the mutants displayed a strikingly different contribution to polymerization activity (Fig. 3B). The two assays gave consistent results: The mutation of TOGs 3/4 and TOG 5 had marginal effects on activity. TOG 1 and TOG 2 contributed strongly to the activity; and the double mutation of TOG 1 and 2 resulted in a protein with minimal polymerase activity.

Polymerase Activity Correlates with Binding to Free Tubulin Dimers. Considering that mutation of all TOG domains in the full length protein prevented interaction with free tubulin dimers, we wanted to see how competent our array of mutants were to bind free tubulin. As was done for the wild-type GFP-tagged protein, we determined this by size exclusion chromatography. As a measure of tubulin binding, we computed amount of tubulin bound per XMAP215 (Figs. S1 and S2). In order to compare tubulin binding to growth, we determined the \( V_{\text{max}} \) for all mutants by repeating the growth experiments at saturating XMAP215 concentrations (200 and 400 nM). Their abilities to bind tubulin fell in a range between that of the wild-type protein and the TOG1-5AA mutant (Fig. S1). In fact, the amount of tubulin bound per XMAP215 was proportional to the polymerase activity (Fig. 3C). Therefore, all TOG domains contribute to both the affinity of XMAP215 for the tubulin dimer and polymerization activity, suggesting that the affinity for tubulin plays an important role in the polymerization mechanism (see Discussion).

Efficiency of Polymerase Activity Increases with Increasing Lattice Affinity. Since TOGs 1 and 2 showed the most significant contribution to activity, we asked whether they were sufficient to promote microtubule growth. We expressed an XMAP215 fragment containing just TOGs 1 and 2 in *Escherichia coli*. This fragment had little polymerase activity at 200 nM protein, where we see maximal growth with wild-type protein (Fig. 3A). We therefore attempted to determine what features displayed by the wild-type protein were absent with the TOG12 fragment. The TOG12 fragment was still able to bind tubulin dimers (Fig. 4A) and microtubule ends (Fig. 4D); however, it had a severely reduced affinity for the microtubule lattice (Fig. 4B). We therefore tested whether addition of a microtubule-binding domain would enhance the
activity of the TOG12 fragment at lower concentrations. We decided to use the K-loop of the kinesin KIF1A to target the TOG12 fragment to the microtubule lattice. We chose this loop because it is a simple basic region that has been reported to effectively target KIF1A and another kinesin, MCAK, to the microtubule lattice (22, 23), and we wanted to exclude other
activities that were potentially present in the region surrounding the native microtubule-lattice-binding domain. Indeed, addition of one K-loop increased the affinity of the TOG12 fragment to the microtubule lattice; addition of three repeats of this domain further increased microtubule-binding activity (Fig. 4B), similar to that of the wild-type protein (see below). We then assayed the ability of these fusion proteins to promote microtubule growth. While the TOG12 fragment alone showed activity at or above 400 nM protein, the TOG12+ and TOG12+++ were active at much lower concentrations. Strikingly, all fragments appear to have a maximum growth rate of approximately 3 µm/min but reach this maximum at very different protein concentrations (Fig. 4C). Furthermore, their activities correspond very well with the fragments’ affinities for the microtubule lattice. These experiments define a minimal “bonsai” polymerase, namely a TOG12 fragment with a strong microtubule-binding domain, which behaves very similar to the wild-type protein. It binds tubulin and the microtubule lattice, promotes fast growth in the low nM range, and is able to track growing microtubule tips (Fig. 4E and Movie S1).

The XMAP215 Microtubule-Lattice-Binding Domain Lies Between TOG4 and TOG5. Because the TOG12 fragment depended on a microtubule-lattice-binding domain to function, we suspected that the native XMAP215 has a microtubule-lattice-binding domain. A region with high affinity (K_D < 1 µM) for microtubules was mapped to a region between residues 1150 and 1325 (13, 24). This region includes part of a region between TOG4 and TOG5 as well as part of TOG5 itself. We wanted to know if TOG5 or any of the TOGs are involved in lattice binding. A fragment containing TOG1-4 (residues 1–1081) bound poorly to the microtubule lattice, consistent with published observations (Figure 5). We made an additional fragment containing the region up to TOG5 (residues 1–1235); it bound microtubules similar to wild type (Fig. 5). Taken together with the published analyses, our experiments suggest that the microtubule-binding domain resides in the region between residues 1150 and 1235, a region that excludes TOG5.

This region is basic, with a predicted pI of 9.8. This is consistent with what is seen in XMAP215 homologues. The *Saccharomyces cerevisiae* homologue of XMAP215, Stu2, has a basic linker after the TOG domains, which has been shown to bind to the microtubule lattice (17, 21). This basic region is also found in *Schizosaccharomyces pombe* Dis1 (25). This region combined with the TOG domains cooperate to promote robust microtubule growth at nanomolar concentrations of protein.

**Discussion**

We have shown that the ability of XMAP215 to efficiently catalyze the incorporation of tubulin dimers into a microtubule is dependent on tubulin binding and microtubule-lattice-binding. We further demonstrate that these activities are mediated by functionally distinct domains. Multiple TOG domains are necessary to increase affinity for the tubulin dimer. Mutants in which these interactions are disrupted are able to efficiently target the microtubule lattice but are impaired in their capacity to promote the incorporation of tubulin dimers into a growing microtubule end. We have also identified a microtubule-lattice-binding domain on XMAP215, localized between TOGs 4 and 5. Deletion of this basic region strongly inhibits the association of XMAP215 with the microtubule lattice. However, protein fragments lacking this domain are still able to promote robust microtubule growth when artificially targeted to the microtubule lattice.

The microtubule plus end can be thought of as an enzyme for the incorporation of tubulin. This enzyme is inefficient, however, because the growth rate in pure tubulin is well below the diffusion limit: The association rate of GTP-tubulin for the individual protofilament plus ends is only 0.3 µM^{-1} s^{-1}, about 20 times smaller than that of ATP-actin for individual protofilament barbed ends in an actin filament (26). It is likely that only a fraction of the tubulin dimers that collide with the plus end become stably incorporated into the microtubule lattice. XMAP215 can be thought of as a nonessential activator of the microtubule end that increases the fraction of tubulin dimers that successfully incorporate into the microtubule lattice (14, 27). When sufficient XMAP215 is added to saturate the plus end, the association rate is increased fivefold to 1.5 µM^{-1} s^{-1} (per protofilament plus end).

In this work, we demonstrate that removal of functional TOG domains affects the ability of XMAP215 to increase the association rate for tubulin to the microtubule plus end. Accordingly, all of the TOG domain point mutants we described showed a lowered maximal growth rate as well as an association rate that falls between 0.3 and 1.5 µM^{-1} s^{-1} per protofilament end. At a fixed tubulin concentration, addition of increasing amounts of XMAP215 resulted in a dose response that achieved maximal growth rate at approximately 200 nM protein, suggesting that the plus ends are saturated with XMAP215 at this concentration. All of the point mutants reached their maximal growth rate at approximately 200 nM protein, consistent with the idea that these proteins are able to bind to the microtubule lattice and target to the plus end similar to the wild-type protein. We therefore conclude that the TOG domains and the tubulin affinity they confer determine the maximal growth rate (ν_{max}) at any fixed tubulin concentration and the decreased capacity for promoting microtubule growth observed with these mutants can be attributed to the fact that ends are saturated with a less effective polymerase (Fig. 6A).

XMAP215 requires a region between TOGs 4 and 5 to target to the microtubule lattice. This positively charged linker may bind to the E-hooks of microtubules, because it has been shown that XMAP215 binds poorly to microtubules whose E-hooks have been removed by subtilisin treatment (14). Removal of the microtubule-binding domain affects the ability of the TOG domains of XMAP215 to work at lower concentrations. The processivity of XMAP215 at the microtubule plus end can be attributed to a combination of tubulin binding and incorporation into the lattice followed by lattice diffusion to the new end via the interaction
Fig. 6. XMAP215 as a catalyst. (A) Mutation or removal of TOG domains result in mutants that have lowered maximal growth rates ($v_{\text{max}}$) at any fixed tubulin concentration. The graph shows theoretical dose response of a protein with increasing affinities for the tubulin dimer that lead to increasing affinities for the transition state (compared to the microtubule end alone): $\alpha = 2$ in red, $\alpha = 5$ in green, and $\alpha = 10$ in blue. $\beta = 1$, $\gamma = 0.1 K_1$ (see SI Text). (B) Mutation or removal of the microtubule-binding domain in XMAP215 results in constructs that have the same $v_{\text{max}}$ but a higher $K_p$. The graph shows theoretical dose response of a protein with a constant $v_{\text{max}}$ and an altered $K_p$ for microtubule-lattice binding: 4x reduced in red, 2x reduced in green, not reduced in blue. $\beta = 1$, $\gamma = 0.1 K_1$ (see SI Text). (C) Model of TOG12$^{+++}$ on the end plus a microtubule. (1) Diffusion to the end via the lattice-binding domain. (2) TOG12$^{+++}$ stabilizes the incoming dimer. (3) TOG12$^{+++}$ remains bound to the incorporated dimer. (4) Release of the dimer. The transitions between each state are described by the reaction scheme in the SI Text.
The GFP tag was introduced on a Nott fragment into the 3′ end after the microtubule-binding domain.

**Protein Expression and Purification.** Full length XMAP215, XMAP215-GFP, full length point mutants, TOG1-4GFP, TOG1-4GFP, ΔTOG1-4GFP, and ΔTOG1-4GFP were expressed in SF+ cells using the Bac-to-Bac system from Invitrogen essentially as described previously (14) except baculovirus infected insect cell (BIIC) stocks were used (31) (see SI Text). All remaining constructs were expressed in E. coli BL21 with plasmid pRARE (see SI Text).

**Tubulin and Microtubule Preparation.** Porcine brain tubulin was purified as described (32). Labeling of cyclic tubulin with Alexa Fluor 488 or TAMRA (Invitrogen) was performed as described (33). GMCPPP microtubules were grown as described (34).

**Imaging.** The total-internal-reflection fluorescence imaging was performed with a setup described previously (14, 34, 35). The setup incorporates an Andor DV887 IXon camera on a Zeiss Axiovert 200 M microscope using a Zeiss 100X/1.45 a Plan-FLUAR objective. Standard filter sets were used to visualize the GFP tag was introduced on a NotI fragment into the 3′ end after the microtubule-binding domain.

**Assay Conditions.** The preparation of silanized cover glasses and perfusion chambers was previously described (14, 34, 35). Reaction conditions were first rinsed with BRB80: 80 mM PIPES at pH 6.9, 1 mM MgCl2, and 1 mM EGTA. Reaction chambers were incubated with either 1% antihorodrome antibody (Invitrogen) or 50 µg/mL neutrophilin (Sigma) in BRB80 for 5 min, followed by 1% pluronic F127 (Sigma) in BRB80 for 5 min, and finally rhodamine-labeled or rhodamine and biotinylated, GMCPPP-stabilized microtubule seeds for 15 min. Channels were washed once with BRB80 and once with imaging buffer (IB): BRB80 supplemented with 75 mM KCl, 15 mM NaCl, 1 mM MgCl2, 1 mM EGTA, 0.1% Tween20, 1 mM DTT. XMAP215 (5.7 µM) and tubulin (14.7 µM) or the equivalent buffer in case of single protein injection were mixed with 0.2 mM GTP in 50 µL total volume, incubated for 10 min on ice and then injected onto the Tosh TS KgelG5000PWXL size exclusion column. For the TOG1 binding experiment, 15 µM TOG1 and 15 µM tubulin were used in 50 µL total volume.

**Size Exclusion Chromatography.** Size exclusion chromatography was carried out similar to ref. 14. Briefly, a Tosoh TS KgelG5000PWXL column was equilibrated in 25 mM TrisHCl pH 7.5, 75 mM NaCl, 1 mM MgCl2, 1 mM EGTA, 0.1% Tween20, 1 mM DTT. XMAP215 (5.7 µM) and tubulin (14.7 µM) or the equivalent buffer in case of single protein injection were mixed with 0.2 mM GTP in 50 µL total volume, incubated for 10 min on ice and then injected onto the Tosh TS KgelG5000PWXL size exclusion column. For the TOG1 binding experiment, 15 µM TOG1 and 15 µM tubulin were used in 50 µL total volume.

**Data Analysis.** Microtubule growth measurements were performed in Meta morph (Universal Imaging). Images were processed using Metamorph and Image J. Curve fitting was done in OriginPro (Origin Lab). Tubulin binding was determined using the heights of the XMAP215, XMAP215:Tubulin and Tubulin peaks (Fig. 52).

**ACKNOWLEDGMENTS.** We thank J. Al-Bassam and S. Harrison for helpful discussions; D. Drechsel, B. Borgonovo, and R. Lemaître for advice and technical assistance; and C. Gell for help with microscopy. We thank members of the Hyman and Howard laboratories for advice and discussions. P.O.W. was supported by a European Molecular Biology Organization long-term fellowship. J.H.S. was supported by the National Institutes of Health National Research Service Award program and the Deutsche Forschungsgemeinschaft, G.B.J. acknowledges support from the Natural Sciences and Engineering Research Council of Canada (Grant 372593). M.Z. is supported by the International Human Frontier Science Program Organization. This work was funded by the Max Planck Society.