Mutational Analysis of Op18/Stathmin-Tubulin-interacting Surfaces
BINDING COOPERATIVITY CONTROLS TUBULIN GTP HYDROLYSIS IN THE TERNARY COMPLEX*

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Oncoprotein 18 (Op18) is a microtubule regulator that forms a ternary complex with two tubulin heterodimers. Dispersed regions of Op18 are involved in two-site cooperative binding and subsequent modulation of tubulin GTPase activity. Here we have analyzed specific determinants of Op18 that govern both stoichiometry and positive cooperativity in tubulin binding and consequent stimulatory and inhibitory effects on tubulin GTPase activity. The data revealed that the central and C-terminal regions of Op18 contain overlapping binding-motifs contacting both tubulin heterodimers, suggesting that these regions of Op18 are wedged into the previously noted 1-nm gap between the two longitudinally arranged tubulin heterodimers. Both the N- and C-terminal flanks adjacent to the central region are involved in stabilizing the ternary complex, but only the C-terminal flank does so by imposing positive binding cooperativity. Within the C-terminal flank, deletion of a 7-amino acid region attenuated positive binding cooperativity and resulted in a switch from stimulation to inhibition of tubulin GTP hydrolysis. This switch can be explained by attenuated binding cooperativity, because Op18 under these conditions may block longitudinal contact surfaces of single tubulins with consequent interference of tubulin-tubulin interaction-dependent GTP hydrolysis. Together, our results suggest that Op18 links two tubulin heterodimers via longitudinal contact surfaces to form a ternary GTPase productive complex.

Microtubules (MTs) are dynamic polymers of αβ-tubulin heterodimers and are required for a variety of cellular processes such as assembly of the mitotic spindle and vesicular transport. Op18 (also termed oncoprotein 18 or stathmin) is a cytosolic protein, which acts to destabilize the MT network. The activity of Op18 is turned off by phosphorylation at four Ser residues in response to multiple signal-transducing protein kinase systems during interphase and cell cycle-regulated protein kinases during mitosis (for review, see Ref. 1). Prevention of phosphorylation-inactivation by mutations of kinase target sites of Op18 results in destabilization of the mitospide and a consequent block in cell division (2, 3), which argues against an active role of Op18 during mitosis. Rather, regulated phosphorylation inactivation of Op18 in response to receptor-stimulated kinase systems suggests that the primary role of Op18 is to modulate the MT system in response to external signals during the interphase of the cell cycle (4, 5).

MTs are continuously rearranging, and most individual MTs are either growing or shrinking with transitions between the two states, a phenomena termed dynamic instability (for review, see Ref. 6). The β-tubulin subunit of the heterodimer contains an exchangeable GTP site (E-site), and MTs utilize polymerization-induced GTP hydrolysis to generate dynamic instability. The tip of a polymerizing MT is thought to contain a stabilizing cap of GTP-tubulin, the loss of which results in depolymerization, i.e. a catastrophe.

Two families of MT destabilizing proteins, represented by XKCM1 and Op18, have been shown to promote MT catastrophes (7–10). XKCM1 acts by physically disrupting the GTP-tubulin cap in an ATP-dependent cycle, but the mechanism by which Op18 promotes catastrophes is still undefined. However, based on the findings of (i) MT plus-end specificity of Op18 catastrophe promotion, (ii) blocking of catastrophe promotion by capping of MTs with a non-hydrolyzable GTP analog (10), and (iii) the ability of Op18 to stimulate GTPase activity of Op18-tubulin complexes (11). A prevalent view is that Op18 may promote catastrophes by stimulating GTP hydrolysis at the plus-end of MTs (for review, see Refs. 12, 13).

Op18 forms a ternary complex with two tubulins and this has been thought to be critical for the mechanism by which Op18 destabilizes MTs (9, 14). Two recent reports have given important insights into the structure of the tubulin-Op18 complex. First, digital image analysis of electron micrographs showed that Op18 links two longitudinally arranged but slightly separated tubulin heterodimers in a protofilament-like fashion (15). Second, mass spectroscopy analysis of chemically cross-linked Op18-tubulin peptides identified contact points between both the central and C-terminal part of Op18 and helix 10 on 32 α-tubulin, a helix located at the surface where longitudinal contacts are made in MTs (16). In both reports it was proposed that Op18 is an extended α-helical protein with separated binding regions for each heterodimer; one in the N-terminal/central part and one in the C-terminal part of the protein. However, two mutually exclusive models of Op18 linkage of the two heterodimers were proposed. Thus, whereas Steinmetz et al. (15) proposed that Op18 linked the two longitudinally arranged heterodimers by extended binding along lateral surfaces, Wallon et al. (16) proposed that each one of two distinct tubulin binding regions of Op18 binds one α-tubulin of the heterodimers via longitudinal contact surfaces, thereby linking the two tubulin heterodimers in a “head-to-head” fashion.

To understand the mechanisms by which Op18 destabilizes MTs, it is important to test these two models and to elucidate...
how Op18-tubulin complex formation stimulates GTP hydrolysis. Here we present evidence that essential contact points for each one of the two tubulin heterodimers in the ternary complex are overlapping within the central region of Op18, and that these contact points are in the vicinity of longitudinally interacting surfaces of tubulin. Our data suggest a third model for Op18-tubulin interactions where Op18 lies between the two longitudinally arranged heterodimers. This model is consistent with the experimental evidence from the two reports discussed above and resolves the mutual incompatibility between the two previously proposed models. Moreover, taken together with previous studies, we present evidence that clearly dissociate stimulation of GTPase activity, which occurs autonomously within the ternary Op18-tubulin complex, from the catastroph-promoting activity of Op18, which is likely to involve interactions with tubulin surfaces exposed at the MT tip.

MATERIALS AND METHODS

DNA Constructs, Expression, and Purification of Recombinant Proteins—Construction of C-terminal-truncated Op18 derivatives with sequences encoding amino acids 100–147 deleted (Op18-Δ100–147) and 90–147 deleted (Op18-Δ90–147) was performed (11, 17). Op18-Δ139–147, Op18-Δ130–147, Op18-Δ116–147, and Op18-Δ80–147 were constructed using an analogous PCR-based strategy and the unique primers: Δ139–147, 5′-CCGCCGCTTACGCTCCTGTT-CTTGGATTCCTGCGC-3′; Δ130–147, 5′-CCGCCGCGTCAGTGGCGG-CTGTTAATCTTTCGCG-3′; Δ116–147, 5′-CCGGGCGCTTCGGAATGCGGGTTCCGCTCTGCGATTTA-3′; and Δ80–147, 5′-CCGGCGGCTAGTGCCGCGG-CTGCGGCGGCTAGTGCCGCGG-3′. Each primer contains a silent hgl/SFJ site at the 5′-end and was used in conjunction with the T7 primer from pBluescript SK(+) (Strategene). Full-length Op18 cDNA was carried on pBluescript as template to amplify PCR fragments corresponding to each of the coding regions of C-terminal-truncated Op18 derivatives (119–147), 5′-TCCCGCTTTGGCGGCCGCTTGGGCCTCGCGATTCTCTTTA-3′ (SacI site introduced with annealed oligonucleotides encoding amino acid residues 116 to 130 of Op18, a corresponding fragment of Op18 cDNA in pGEX 4T-1 (18). Op18-Δ(116–147), was constructed by overlap-PCR using the primers 5′-CTTTGTTCTTCCGCAGTTACGCTCCTGCGC-3′, to gethel with the T7 and T3 primers of pBluescript. Op18-Δ(119–147), Op18-Δ(123–147), and Op18-Δ(127–147) were constructed by extending Op18-Δ(5′-SfI116) from the SFJ site with annealed oligonucleotides encoding C-terminal amino acid residues following the two consecutive stop codons: Δ119–147, 5′-CCGCCGCTTGCCGCAGTACATGCAACA-3′ and 5′-GGGCTTATGCTGGCCGAGCTGGCCTTG-3′; Δ123–147, 5′-GGGCTTATGCTGGCCGAGCTGGCCTTG-3′; Δ127–147, 5′-GGGCTTATGCTGGCCGAGCTGGCCTTG-3′. Coding regions of C-terminal-truncated Op18 derivatives were excised as NcoI to NotI fragments and used to replace the corresponding fragment of Op18 cDNA in pGEX 4T-1 (18).

Construction of the N-terminal-truncated derivatives Op18-Δ(5′–4)–45 and Op18-Δ(5′–5)–55 have been previously described (19). It should be noted that Op18-Δ(5′–4)–45 was termed Op18-Δ(5′–4)–45 in its original description, but these derivatives are identical because both residues 4 and 46 are serines. Op18-Δ(5′–4)–45 and Op18-Δ(5′–5)–52 were constructed by PCR using the primers Δ4–62, 5′-TCCCGAGCTCAGATGAGCTGAGCTGAAGGCGTGCGTTC-3′ and Δ5–72, 5′-TCCCGAGCTCAGATGAGCTGAGCTGAAGGCGTGCGTTC-3′ with the T7 primer from pBluescript SK(+) as second primer and FLAG epitope-tagged Op18 as template (17). The primers introduce a silent SacI site in the 5′-end. The PCR fragments were excised as SacI to BamHI fragments and inserted into the corresponding sites of pET3d Op18-Δ(5′–4)–45 (10). The sequence coding for PCR-generated fragments were confirmed by nucleotide sequence analysis using ABI PRISM dye terminator cycle sequencing kit from PE Biosystems.

GST fusion proteins were expressed and purified on glutathione-Sepharose 4B beads as recommended by the manufacturer (Amersham Pharmacia Biotech). FLAG-tagged Op18 derivatives were expressed and purified as described previously (19). Purified recombinant proteins were routinely analyzed by SDS-polyacrylamide gel electrophoresis to confirm purity and to confirm that each truncated Op18 derivative migrated corresponding to the predicted molecular weight.

Assays of Tubulin GTPase Activity—Assay of tubulin GTPase activity was performed in PEM buffer adjusted to pH 6.5 with KOH (80 mM piperazine-N,N′-bis[2-ethanesulfonic acid], 1 mM EGTA, 4 mM MgCl2) containing 5 mM AMP-PNP, to inhibit nonspecific ATPase activity as described (11). In brief, tubulin was incubated with [α-32P]GTP, tubulin-[α-32P]GTP complexes recovered by centrifugation through a desalting column (P-30 Micro Bio-Spin, Bio-Rad) and single-turnover GTP hydrolysis was followed at 37 °C. Control experiments showed that the Op18 preparations used neither bound nor hydrolyzed [α-32P]GTP. Nucleotide hydrolysis was quantitated by ascending chromatography as described (20).

Analysis of Op18-Tubulin Binding—Op18-tubulin equilibrium binding experiments were in principle performed as previously reported (18) but with modifications to increase detection of low-affinity binding. In brief, N-terminal GST-tagged or C-terminal FLAG-tagged Op18 derivatives (2 μM) and tubulin (0.8–42 μM) were mixed and allowed to associate for 15 min on ice. Op18-tubulin mixes (48 μl) were added to glutathione or anti-FLAG antibody-coupled-Sepharose beads (12 μl) and incubated for 15 min at 8 °C to capture Op18-tubulin complexes. Rapid separation of Op18-tubulin complexes bound to glutathione or anti-FLAG antibody-coupled beads was obtained by applying the bead anti-GST-domain bound to the caps of 1.5-ml Eppendorf tubes prepared with a bottom layer of 150 μM of PEM complemented with 27% sucrose, 17% glycerol, pH 6.0. Samples were centrifuged shortly (30 s, 21,000 × g) to separate bead-bound from -free material. The buffers, and centrifugation steps described above allowed increased detection of low-affinity binding between truncated Op18 and tubulin as compared with our previous protocol (18).

To allow simultaneous quantification of Op18 and tubulin in the same sample, tubulin was labeled with [α-32P]GTP and Op18 was labeled with [35S]methionine. There are two major benefits to this strategy. First, the amount of GST-Ot18/Op18F present in the fraction of free tubulin after separation from the beads (about 30% of total Op18) can be compensated for in each data point, and second, only biologically active tubulin (i.e. GTP-bound) tubulin was detected in the fraction of free tubulin. Additionally, the labeling of nonspecific tubulin binding was around 5% of the amount of free tubulin and was subtracted from the data presented. To calculate Op18-tubulin binding parameters, data points from equilibrium binding experiments were fitted either to a one affinity binding model (hyperbola) or to a previously described model assuming two-site positive cooperativity in binding (21). Written in the form of a binding curve, a two-site positive cooperativity model has the form,

$$B = \frac{B_{\text{max}}}{1 + \frac{1}{1 + \frac{F}{K_{d1}}}} \times \frac{2 F}{K_{d1} \times K_{d2}} \times \frac{1}{K_{d2}}$$

(1)
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Fig. 1. Op18 truncation derivatives. Helices are denoted according to Wallon et al. (16); helix 1 (residues 46–87), helix 2 (residues 96–134), a putative N-terminal α-helical region (?) and a polyproline helix (PPII, residues 33–43). Phosphorylation sites are indicated with P (Ser-16, -25, -38 and -63). For stability of truncated proteins, 3–4 amino acids of the N terminus and 2 amino acids of the C terminus of Op18 were preserved. The derivatives are epitope-tagged in the opposite end relative to the truncation. C-terminal truncations are tagged with GST and N-terminal truncations with the FLAG epitope.

erodimers, which corresponds to the approximate diameter of an α-helix. To determine whether Op18 links two tubulin heterodimers by independent or overlapping binding regions, we determined the stoichiometry of tubulin binding after consecutive truncations of Op18 from either the N or the C terminus. To facilitate quantitation of binding stoichiometry of truncated Op18 derivatives with impaired tubulin affinity, binding conditions were set to maximize affinity and to minimize dissociation during separation of bound complexes (see “Materials and Methods”). The truncation mutants generated for this purpose are outlined in Fig. 1 together with a secondary structure representation of Op18 according to Wallon et al. (16). To avoid the fact that N- and C-terminal truncations indirectly obstruct binding by altering the position of the affinity tag used for separation of Op18-tubulin complexes, either the N-terminal GST-tag or the C-terminal FLAG-tag was introduced at opposite ends relative to truncations (see Fig. 1). Tubulin binding affinity and stoichiometry of full-length Op18 (Op18-wt) fused to either of these two affinity tags were essentially the same (Fig. 2, insets in B and D). In agreement with our previous study (18), Scatchard conversion of binding data showed that tubulin binding to Op18-wt is a complex process with nonlinear data-point distribution typical for positive cooperativity in binding. Analysis of C-terminal-truncated Op18 revealed that truncation up to residue 90 still allowed tubulin binding, albeit with poor affinity. Importantly, Scatchard analysis of binding data indicated that both Op18-Δ(90–147) and Op18-Δ(100–147) are still capable of binding two tubulin heterodimers, but the data-point distributions suggest non-cooperative binding (see Fig. 2 figure legend for $B_{max}$ values). However, if C-terminal truncations extend into helix 1 (see Op18-Δ(80–147)), the binding affinity is too low for interpretable binding data. We conclude that all C-terminal-truncated Op18 derivatives that bind tubulin with estimable affinities are capable of binding two heterodimers.

The corresponding deletion analysis from the N terminus reveals that removal of more than 45 amino acids resulted in a major loss of tubulin binding affinity (compare Op18-Δ(4–45) and Op18-Δ(5–55) in Fig. 2, C and D). However, the Op18-Δ(5–55) mutant, which has 10 amino acids of helix 1 deleted, was still capable of binding two tubulin heterodimers. Upon removal of an additional 7 or 17 amino acids from the N terminus, binding affinities became too low for interpretable binding data. Scatchard conversion of Op18-Δ(4–45) binding data revealed data-point distribution typical for positive cooperativity in binding, indicating that the N terminus is not important for the observed cooperativity in tubulin binding (Fig. 2D).

The data above show that N- or C-terminal truncations that extend into helix 1 cause the most drastic drops in tubulin binding affinity. However, as shown in Fig. 2C, helix 1 is clearly a very poor tubulin binder by itself as revealed by analysis of Op18-Δ(4–45), Δ(100–147)-F (termed H1-F). It seems unlikely that this result was biased by the use of the C-terminal FLAG-tag for separation of complexes, because the FLAG-tag can be efficiently used for separation of tubulin-Op18-Δ(100–147)-F complexes (data not shown and Ref. 18). Moreover, our result is in line with a recent report demonstrating that truncation of both the N- and C-terminal flanks of Op18 abolishes tubulin binding as determined by size exclusion chromatography (23).

Taken together, because N- and C-terminal-truncated Op18 either bound two heterodimers of tubulin or was incapable of binding tubulin with an estimable affinity, the data demonstrate that essential parts of both tubulin binding sites of Op18 are overlapping within the central α-helical part of Op18. Given overlapping binding sites and the previously noted 1-nm gap between longitudinally arranged tubulin heterodimers in complex with Op18 (15), the simplest interpretation of binding data is that at least helix 1 of Op18 is inserted between the heterodimers.

The C-terminal Region of Op18 Determines Cooperativity in Tubulin Binding—We have recently shown that Op18 binds tubulin according to a two-site positive cooperative model, i.e. Op18 binding to the first tubulin creates a second tubulin binding site of much higher affinity (18). The binding data above show that both the N- and C-terminal regions flanking helix 1 are important for stabilization of the ternary Op18-tubulin complex. Interestingly, whereas derivatives with N-terminal truncations retained positive cooperativity, C-terminal truncations appear to bind tubulin without appreciable cooperativity. It was therefore of interest to search for a specific region within the C terminus that may impose positive cooperativity.

To determine positive cooperativity in a series of successive C-terminal truncation derivatives of Op18, we excluded glycerol from the binding buffer. This results in lower tubulin binding affinities as compared with the data shown in Fig. 2, but two-site positive cooperativity can be more accurately resolved (18). The results in Fig. 3 show that whereas truncation of residues 139–147 had little effect, truncation of residues 130–147 caused a major decrease in tubulin binding affinity.
As predicted from the data in Fig. 2, Scatchard analysis revealed that all Op18 derivatives tested were capable of binding close to two heterodimers (Fig. 3, C and D). Importantly, however, Scatchard analysis of binding, which reveals cooperativity by a pronounced curvature, demonstrates dramatic differences between Op18 truncation derivatives on the level of binding cooperativity.

By fitting a cooperative binding model to the data, the first ($K_{d1}$) and second ($K_{d2}$) affinities were resolved. The ratio of $K_{d1}$ over $K_{d2}$ is 1 in a situation of no cooperativity, whereas increasing positive cooperativity results in an increased ratio. The ratio of affinities presented in Table I, pinpoints 7 amino acids (residues 123–129) in the C terminus of Op18 that are critical for positive cooperativity in tubulin binding. It is evi-
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The data in Fig. 3 were used to calculate equilibrium dissociation constants according to a two-site positive cooperativity model. The degree of cooperativity is presented as the ratio between $K_{d1}$ and $K_{d2}$ (1.0 = no cooperative binding). Data were also fitted to a one affinity model and fits were compared as described in "Materials and Methods." Op18 derivatives giving a significant fit to the cooperativity model are indicated (***, p < 0.001; **, p < 0.01; ns, not significant). Free tubulin concentration at half-saturation of Op18 was estimated from the binding curves in Fig. 3.

| F-test | $K_{d1}$ $\mu M$ | $K_{d2}$ $\mu M$ | $K_{d1}/K_{d2}$ | Free [tubulin] $\mu M$ at half-saturation of Op18 |
|--------|------------------|------------------|------------------|-----------------------------------------------|
| *** Op18-wt | 3.0 ± 0.8 | 0.10 ± 0.03 | 30 | 0.51 |
| *** $\Delta$ (139–147) | 4.0 ± 1.2 | 0.11 ± 0.04 | 38 | 0.67 |
| *** $\Delta$ (130–147) | 34 ± 4.0 | 1.1 ± 0.4 | 31 | 5.8 |
| ** $\Delta$ (127–147) | 24 ± 2.5 | 3.6 ± 0.7 | 6.8 | 9.6 |
| NS $\Delta$ (123–147) | 25 ± 2.0 | 11 ± 3.4 | 2.3 | 17 |
| NS $\Delta$ (100–147) | 21 ± 1.3 | 16 ± 9.0 | 1.4 | 17 |

* Mean ± S.E.

DISCUSSION

Model for Op18 Binding to Tubulin—The present study identifies a 7-amino acid sequence in the C terminus of Op18 that is essential for two-site positive cooperativity in tubulin bind-
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Fig. 4. The cooperativity imposing region of Op18 determines the switch from tubulin GTPase stimulatory to inhibitory activity. A, tubulin (10 μM in PEM, pH 6.5) preloaded with [α-32P]GTP was incubated at 37 °C with 36 μM of the indicated GST-tagged Op18 derivatives. B, tubulin was incubated as in A with graded concentrations of the indicated GST-tagged Op18 derivatives. Initial single-turnover hydrolysis rates were evaluated as described under “Materials and Methods.” Data are mean ± S.E. of duplicate determinations.

Fig. 5. Tubulin concentration dependence of Op18-mediated stimulation and inhibition of GTPase activity. Graded tubulin-[α-32P]GTP concentrations incubated alone or mixed with a 3-fold molar excess of FLAG-tagged Op18-wt or Op18-Δ(100–147), as indicated. Initial hydrolysis rates were evaluated as in Fig. 4. Data are extrapolated toward zero concentration of tubulin to get an estimation of the autonomous component of GTP hydrolysis. Data are mean ± S.E. of duplicate determinations.

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The results from equilibrium binding analysis dissociate the apparent binding affinity from binding cooperativity. For example, it is shown that a short C-terminal region (residues 130–139) mediates about 10-fold increase in the apparent tubulin binding affinity without major effect on binding cooperativity (see Table I). Moreover, our previous study shows that deletions from the N terminus decrease the apparent affinity, although actually increasing positive cooperativity (18). We therefore conclude that cooperativity and the apparent binding affinity are independent parameters regulated via multiple non-essential physically separated regions of Op18. Binding analysis in the present study also shows that essential parts of binding sites for two individual tubulin heterodimers are intimately associated within helix 1. Thus, all truncated Op18 derivatives that bind tubulin with appreciable affinities were shown to be capable of binding two individual tubulins. However, it should be noted that under conditions of low tubulin concentration and excess Op18 used for tubulin GTPase assays, non-cooperative binding by C-terminal-truncated Op18 promotes binding of single tubulins with consequent inhibition of GTPase activity.

Two recent publications have brought insight into tubulin-Op18 interactions. In the first study, contact points between Op18 and tubulin were identified by mass spectrometric analysis of cross-linked complexes and shown to include helix 10 of α-tubulin, a helix involved in longitudinal contacts within the MT lattice (16). In the second study, digital image analysis of electron micrographs of Op18-tubulin complexes reveals that the complex consists of two longitudinally aligned tubulin heterodimers oriented in a slightly kinked protofilament-like fashion (15). The combined information from these two studies together with binding data from our mutant series allow a refined model for the Op18-tubulin complex. Our data reveal that the two tubulin binding sites of Op18 are intimately associated along helix 1 and that binding affinity toward both of the two individual tubulins diminish after stepwise truncations from either the N or C terminus. This suggests that most of the binding determinants of Op18 are situated inbetween the two heterodimers, and that overlapping regions of Op18 contribute to binding of both heterodimers. Such a model is compatible with image analysis of Op18-tubulin complexes, which shows a 1-nm extra separation of the heterodimers than predicted from the MT structure. Most importantly, helix 10 of α-tubulin, which defines a region involved in longitudinal contacts of tubulins has been identified as a major contact point for Op18 helix 1 and 2 (16). This result combined with the present binding analysis of Op18 truncation mutants provides compelling evidence indicating that parts of helix 1 and the C-terminal flanking helix 2 of Op18 are intercalated between the two heterodimers.

Based on the apparent lack of tertiary structure of Op18 in solution, two alternative models on tubulin binding have been proposed so far. In the first model, based on identification of Op18-tubulin contact points, helix 1 and the C-terminal-flanking helix 2 are proposed to each bind one α-tubulin of the heterodimers, independently of each other, and thereby link two tubulin heterodimers in a head to head-like fashion (16). In the second model, based on digital image analysis, it is proposed that an extended Op18 molecule binds along two tubulin heterodimers orientated in a protofilament-like fashion with the N terminus of Op18 serving as a cap for the exposed E-site of one of the heterodimers (15). These two models are clearly very different and mutually exclusive, but with respect to the orientation of the two heterodimers, the proposed protofilament-like fashion of the second model has much stronger support from the presented data. Nevertheless, in both the first and second model, it is suggested that the binding sites on Op18 for the two tubulin heterodimers are separate.

Both models of Op18-tubulin interactions outlined above are incompatible with data from Op18-tubulin binding analysis presented herein. As argued above, the combined evidence by...
us and Wallon et al. (16) demonstrate that at least parts of helix 1 and the C-terminal helix 2 of Op18 are intercalated between the two tubulin heterodimers. Moreover, by employing binding conditions optimized for high-affinity Op18-tubulin interactions (i.e. pH 6.5 in the presence of glycerol), it was shown that all truncated Op18 derivatives that bind tubulin with estimable affinities are at sufficiently high tubulin concentrations capable of binding two individual heterodimers. These binding analyses reveal the importance of the central helix 1. However, this region is by itself not sufficient for substantial binding affinity, which requires either the N- or C-terminal flanks of Op18. Both of these flanking regions of Op18 are competent to stabilize the ternary Op18-tubulin complex independently of each other, but only the C-flank does so by imposing positive binding cooperativity.

**Modulation of Tubulin GTPase Activity by Op18**—Within the MT-polymer, hydrolysis of the E-site bound GTP of β-tubulin is believed to be triggered by a catalytic Glu residue in a loop on the preceding α-tubulin subunit (24). Under non-polymerizing conditions, the same basic tubulin GTPase stimulatory mechanism operates, but the positioning of the catalytic loop is dependent on α-tubulin/β-tubulin collision events (25). It is shown herein that two-site cooperative binding between Op18 and tubulin results in a complex with autonomous GTPase activity, whereas non-cooperative binding of C-terminal-truncated Op18 results in potent inhibition of tubulin-tubulin interaction dependent GTP-hydrolysis.

It has recently been proposed that Op18 stimulates tubulin GTPase activity within a ternary complex by promoting interactions between the E-site of β-tubulin and the catalytic loop on α-tubulin (15). This model was proposed by Steinmetz and co-workers with the assumption that Op18 binds laterally along the longitudinally arranged tubulin heterodimers. However, as discussed above, several independent lines of evidence indicate that Op18 is intercalated between the two heterodimers. This is likely to be relevant for the mechanism behind stimulation and inhibition of GTP hydrolysis and changes an important premise for the model by Steinmetz et al. (15). Moreover, the potent GTPase inhibitory activity of C-terminal-truncated Op18 provides additional evidence against the model. Thus, after mutational disruption of positive binding cooperativity, which favors binding to single tubulins under the conditions used for GTPase assays, Op18 blocks tubulin-tubulin interaction-stimulated GTP hydrolysis (Figs. 4 and 5). Non-cooperative Op18 binding to longitudinal contact surfaces on single tubulins can readily explain this phenomenon, because this would protect either the E-site on β-tubulin or the catalytic loop on α-tubulin, or both, from GTPase-productive interactions. The combined evidence from analysis of tubulin binding and modulation of GTP hydrolysis is difficult to reconcile with the mechanism proposed by Steinmetz et al. (15). It seems, therefore, more likely that Op18 stimulates tubulin GTPase activity by a mechanism that is distinct from GTPase stimulation within the MT lattice.

**Tubulin Binding and Catastrophe Promotion by Op18**—The molecular mechanism by which MT catastrophe-promoting factors such as Op18 or XKCM1 are acting is likely to involve disruption of the stabilizing MT-cap. XKCM1 does this mechanistically by utilizing energy in the form of ATP (8). Op18 on the other hand, which in contrast to XKCM1 only stimulates catastrophes from the plus-end of MTs, utilizes no exogenous energy (for review, see Ref. 26). There are two probable scenarios by which catastrophes could be induced without external energy input; namely (i) stimulation of GTP-hydrolysis at the stabilizing cap at the MT plus-end, and (ii) binding to the exposed longitudinal contact surface at the MT plus-end and thereby preventing growth or altering the symmetry of the cap.

Steinmetz et al. (15) has proposed a model where the Op18 N-terminal flanking region targets Op18 to the MT-end, which stimulates GTP-hydrolysis within the stabilizing cap followed by a catastrophe (15). This model has been tested herein by a detailed structure/function analysis of tubulin binding by Op18 and subsequent modulation of GTP-hydrolysis. The results reveal several lines of evidences that argue against an involvement of GTPase stimulation during catastrophe promotion. First, as discussed above, the observed GTPase stimulation depends on two-site positive binding cooperativity, which brings two tubulin heterodimers into a GTPase active ternary Op18-tubulin complex. The requirement of a ternary complex for GTPase stimulation suggests that the GTPase stimulating activity of Op18 is not directed toward the end of MTs. Second, Op18-mediated stimulation of GTP hydrolysis is very inefficient. Thus, Op18-stimulated hydrolysis requires on average almost an hour per event (see Figs. 4 and 5), whereas in a growing MT with a constant sized GTP cap the hydrolysis rate must be the same as the rate of subunit incorporation, i.e. much less than a second time scale. Finally and most importantly, C-terminal-truncated Op18 has lost the GTPase stimulatory activity and gained an activity that inhibits tubulin GTP hydrolysis. Because such a derivative is still efficient in promoting catastrophes both in vitro and in intact cells (10, 18), our results clearly dissociate the tubulin GTPase stimulatory activity of Op18 from catastrophe promotion.

As discussed above, C-terminal-truncated Op18 has lost positive cooperativity in tubulin binding, which ensures that GTPase productive ternary Op18-tubulin complexes are formed, but retains catastrophe-promoting activity. This provides a clear mechanistic uncoupling of formation of ternary tubulin complexes and catastrophe promotion by Op18. Hence, the functional role of ternary Op18-tubulin complexes and consequent GTPase activation for MT destabilization is unclear. Genetic dissection of specific MT-directed activities both in intact cells and in vitro has revealed at least two functions of Op18. Thus, the N-terminal region is required for catastrophe promotion, whereas the C-terminal region is required to inhibit the MT polymerization rate in vitro and to destabilize MTs in intact cells by an as yet poorly defined mechanism (10, 18). Thus, the C-terminal region that imposes positive cooperativity in tubulin binding is clearly important for some aspects of Op18 function and warrants future investigations.

Op18 is an abundant protein in many cell types, particularly in malignant cells (1). For example, the concentration of endogenous Op18 in the leukemic K562 cell line is about 10 µM, which is sufficient to form ternary tubulin-Op18 complexes with essentially all cellular tubulin, which is present at about 20 µM (18). However, Op18 at these high concentrations clearly does not disrupt cellular MTs, because the ratio of free to polymerized tubulin dimers is normal in K562 as well as other cells expressing high levels of Op18. This suggests that Op18 is very inefficient in sequestering tubulin in intact cells, which could be explained by the decrease in binding affinity observed at cellular pH (i.e. above pH 7) (22). There is also another line of evidence suggesting that tubulin sequestering by Op18 is not an important mechanism for MT destabilization in intact cells. Thus, analysis of both N- and C-terminal-truncated Op18 derivatives in crude cell extracts of transfected K562 cells show that because of the affinity difference between wild type and truncated Op18, endogenous Op18 outcompeted essentially all binding to soluble tubulin by overexpressed truncated Op18 (18). Despite this, efficient MT destabilization as a result of overexpressing the truncated Op18 derivatives was still observed, which provides evidence for tubulin sequestering inde-
pendent MT destabilization in intact cells. However, given the high intracellular Op18 concentrations, it is evident that Op18 is not a potent catastrophe promotor in intact cells and putative MT end-specific interactions must be of sufficiently low affinity to avoid complete disruption of the MT system. It seems also likely that the presence of MT associated proteins in intact cells counteracts MT destabilization by Op18.

The combined results of the present study and the previous study by Wallon et al. (16) clearly indicate that Op18 binds to tubulin surfaces involved in longitudinal contacts. This suggests a model for catastrophe promotion in which low affinity Op18 interactions with the exposed longitudinal surfaces at the tips of MTs will prevent or mis-orient growth of that particular protofilament in the polymer. This would be in line with a recent study on the relation of MT end structure and the regulation of dynamic instability, which suggests that it is the maintenance of the MT-tip structure rather than the GTP-tubulin subunits that gives the important stabilizing property of the cap (27).

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