Molecular Dissection of GTP Exchange and Hydrolysis within the Ternary Complex of Tubulin Heterodimers and Op18/Stathmin Family Members*

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The ubiquitous Op18 and the neural RB3 and SCG10 proteins are members of the oncoprotein18/stathmin family of microtubule regulators. These proteins bind two tubulin heterodimers via two imperfect helical repeats to form a complex of heterodimers aligned head-to-tail. Here we have analyzed GTP exchange and GTP hydrolysis at the exchangeable GTP-binding site (E-site) of tubulin heterodimers in complex with Op18, RB3, or SCG10. These proteins stimulate a low and indistinguishable rate of GTP hydrolysis, and our results show that GTP exchange is blocked at both E-sites of the ternary complex, whereas GTP hydrolysis only occurs at one of the two E-sites. Results from mutational analysis of clusters of hydrophobic residues within the first helical repeat of Op18 suggest that GTP is hydrolyzed at the E-site that is interfaced between the head-to-tail arranged heterodimers, which is consistent with predicted GTPase productive interactions between the two tubulin heterodimers. Our mutational analysis has also indicated that Op18/stathmin family members actively restrain the otherwise potent GTPase productive interactions that are generated by longitudinal interactions within protofilaments. We conclude that tubulin heterodimers in complex with Op18/stathmin family members are subject to allosteric effects that prevent futile cycles of GTP hydrolysis.

The Op18/stathmin family of microtubule regulators includes the ubiquitous cytosolic Op18/stathmin (Op18)1 and the neuronal proteins RB3, SCG10, and SCLIP (for review, see Ref. 1). A characteristic feature of the neural family members is a tandem repeat of Op18 binding along one heterodimer to generate a head-to-tail alignment, with each one of the tandem helical repeats of Op18 binding along one heterodimer to generate a ternary tandem tubulin heterodimer complex, which is stabilized by longitudinal interactions between the two heterodimers (see Fig. 1). As predicted by these stabilizing heterodimer-heterodimer interactions within the complex, Op18 binds two heterodimers according to a two-site positive cooperative model, which strongly favors formation of a ternary complex rather than binary complex formation with a single tubulin heterodimer (5). The 4-A resolution x-ray structure has allowed rejection of alternative models for the ternary complex (6, 7), but was of insufficient resolution to resolve the orientation of the extended Op18 helix. Cross-linking experiments, however, indicate that the N terminus of Op18 is oriented toward the exposed α-tubulin end of the head-to-tail-aligned heterodimers (8) (see Fig. 1).

Microtubules are polymers built up from α/β-tubulin heterodimers, and a characteristic feature of individual microtubules is frequent and stochastic switching between polymerization and depolymerization cycles, a phenomenon termed dynamic instability (for review, see Ref. 9). The β-tubulin subunit of the heterodimer contains an exchangeable GTP-binding site (termed E-site), and microtubules utilize polymerization-induced GTP hydrolysis to generate dynamic instability. The mechanism of GTP hydrolysis involves longitudinal interactions of polymerizing α/β heterodimers via a catalytic loop located on the α-tubulin that interacts with the E-site of the adjacent β-tubulin subunit (10). The tip of a polymerizing microtubule contains a stabilizing cap of GTP-tubulin, the loss of which results in a catastrophe (i.e. transition from a growing to a shrinking polymer).

Op18 was originally described as being a specific catastrophe-promoting protein (11), but this was subsequently challenged by a study claiming that Op18 acts solely by sequestering tubulin heterodimers (12, 13). This controversy was resolved by a study demonstrating that Op18 mediates both catastrophe promotion and sequestration of tubulin and that detection of these activities depends on buffer conditions (14). It was also shown in the same study that the non-helical N-terminal region is essential for catastrophe-promoting activity, whereas the extended helical part of Op18 containing the two imperfect repeats is necessary and sufficient for tubulin-sequestering activity. Recent studies involving ectopic expression of truncated/mutated Op18 derivatives in human cell lines have indicated the significance of the functional dichotomy of Op18 that is observed in vitro (5, 15, 16).

How Op18 promotes catastrophes is still obscure, but given that the second helical repeat is dispensable, binding studies (5, 7) combined with recent structural insights (4) allow the conclusion that the mechanism does not involve the two-site...
positive cooperative binding activity of Op18 that is required for efficient formation of ternary tandem tubulin heterodimer complexes (see Fig. 1). Moreover, such recent structural insights readily explain the requirement for the second helical repeat in generating an Op18-tubulin heterodimer complex of sufficient stability to exert tubulin-sequestering activity. The two-site positive cooperative binding activity of Op18 also has additional functional consequences that are distinct from tubulin sequestration, such as inhibition of GTP exchange and stimulation of autonomous low rate GTP hydrolysis within the ternary complex (5, 7, 17). Truncation of the second helical repeat of Op18, which does not alter the catastrophe-promoting activity, terminates stimulation of GTP hydrolysis (5, 7). Hence, stimulation of tubulin GTP hydrolysis by Op18 depends strictly on the cooperative tubulin heterodimer binding activity that mediates the tubulin head-to-tail configuration of the complex.

As outlined above, the cooperative Op18 binding of two tubulin heterodimers inhibits GTP exchange and stimulates autonomous low rate GTP hydrolysis within the ternary complex. In the present study, we have functionally dissected Op18 and the homologous RB3 and SCG10 proteins with the aim of understanding the significance of these GTP-regulatory events.

MATERIALS AND METHODS

DNA Constructs, Expression and Purification of Recombinant Proteins—Wild-type Op18 and an Op18 derivative with the non-helical N-terminal 45 amino acids deleted (termed Op18-R1 + 2 because it contains the tandem repeats 1 and 2 of the extended helix; see Fig. 1) have been described (5). Both of these Op18 derivatives contained an eight-amino acid C-terminal FLAG epitope. Soluble SCG10 (sSCG10), with the hydrophobic N-terminal 30-amino acid membrane targeting region removed, was prepared by a PCR strategy using a full-length cDNA clone of SCG10 (SCG10-8.6, (18), a gift from Dr. N. Mori) as a template. The eight-amino acid C-terminal FLAG epitope was introduced by a 24-nucleotide insertion before the stop codon in the 3′ PCR primer. An N-terminally truncated SCG10 derivative (termed SCG10-R1 + 2), which corresponded to Op18-R1 + 2 (see Fig. 1), was prepared by a PCR strategy using sSCG10 DNA as a template. As part of the cloning strategy for truncated proteins, the first three amino acids of the N terminus of Op18 were added to the N terminus of Op18-R1 + 2 and SCG10-R1 + 2 derivatives. The soluble form of RB3 (sRB3) and the RB3-R1 + 2 derivative were constructed using an identical PCR strategy to that described above for SCG10. An RB3 cDNA, obtained from Research Genetics, Huntsville, AL, was used as a template (GenBank accession number AL534520). The Op18-family derivatives described above were expressed with a six-residue His tag at the N terminus and purified from Escherichia coli using pET-3d expression as described previously (5). Construction of the Op18-pmut1, where the codons for Leu-47, Ile-50, and Leu-54 are exchanged for Ala codons, has been described previously (originally termed Op18-cm1) (19). Op18-pmut2, where the codons for Val-68, Leu-69, and Leu-72 are exchanged for Ala codons, and Op18-pmut3, where the codons for Val-82 and Leu-83 are exchanged for Ala codons, were constructed using a general strategy where mutations were introduced into subfragments of the coding region by overlapping PCR using wild-type Op18 as template. Coding regions of these Op18 derivatives were expressed and purified as glutathione S-transferase (GST) fusion proteins in pGEX 4T-1 (7). The coding sequences of PCR-generated fragments were confirmed by nucleotide sequence analysis using the ABI PRISM dye terminator cycle sequencing kit from PerkinElmer Life Sciences. Purified recombinant proteins were routinely analyzed by SDS-PAGE to confirm the homogeneity and determine the protein concentration of each preparation.

Assays of Tubulin GTPase Activity—Analysis of tubulin GTPase activity was performed in PEM buffer (80 mM piperrazine-N,N′-bis(2-ethanesulfonic acid), 1 mM EGTA, 4 mM MgCl2) containing 17% glycerol adjusted to the indicated pH with NaOH. The buffer also contained 5 mM adenosyl-5′-diphosphate (AMP-PNP; to inhibit nonspecific ATPase activity) as described previously (7). In brief, tubulin (TL238, Cytoskeleton, Inc.) was incubated with [γ-32P]GTP, the resulting tubulin-[γ-32P]GTP complexes were 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 bind nor hydrolyze [γ-32P]GTP or cause dissociation of tubulin-bound [α-32P]GTP. Nucleotide hydrolysis was quantified by thin-layer chromatography as described (7), which allows reproducible analysis of less than 0.2% hydrolysis of the [α-32P]GTP.

Determination of Tubulin-GTP Exchange Rates and Plasmon Resonance Experiments—[α-32P]GTP-tubulin (0.8 mol GTP/mol tubulin, ~4 × 10^15 dpm/mol) was incubated at 5 µM in PEM buffer at the stated pH and glycerol concentration) on ice for 10 min in the presence or absence of the indicated Op18-family derivative. The reaction was started by the addition of 2 mM cold GTP at 37 °C, and exchange rates were followed over time by separation of unbound [α-32P]GTP through a desalting column (P-30 Micro Bio-Spin, Bio-Rad). These columns retained more than 99.99% of all non-tubulin bound [α-32P]GTP, whereas the flow-through contained tubulin and Op18 (>95% yield). Twenty microliters of the reaction mixture was sampled per time point. The GTP exchange rates were calculated by quantifying the radioactivity of the samples and the desalting columns. Plasmon resonance competition experiments were carried out on a BIAcore 3000 system with Op18 immobilized by amine coupling on a CM5 chip according to the instructions of the manufacturer. Analyses were performed with the indicated concentrations of tubulin in PEM, pH 6.8, premixed with graded concentrations of Op18 derivatives. The free tubulin concentrations were determined from the plateau levels by comparison with a standard tubulin curve, as described in the BIAcore handbook. Dissociation constants refer to the binding of two tubulin heterodimers.

RESULTS

Modulation of Tubulin GTP Exchange and Hydrolysis by Complex Formation with Op18/Sheathmin Family Members—Complex formation between Op18 and tubulin heterodimers has previously been shown to result in low rate tubulin GTP hydrolysis (5). To determine whether this property is conserved, we analyzed soluble derivatives of two neural members of the Op18/sheathmin family, SCG10 and RB3, in which the membrane-targeting regions were deleted (see sSCG10 and sRB3 in Fig. 1). Single-turnover tubulin GTP hydrolysis was determined at 37 °C by incubation of Op18/sheathmin family derivatives with tubulin at a concentration that was suffi-
cantly low to avoid polymerization. As shown in Fig. 2, all three Op18/stathmin family members tested stimulated tubulin GTP hydrolysis ~3-fold as compared with the basal hydrolysis rate. Moreover, analysis of N-terminally truncated derivatives that only encompass repeats 1 and 2 (see Fig. 1) revealed unaltered tubulin GTPase stimulation (compare Fig. 2, A and B). It should be noted that truncation of the 45 residues at the non-helical N terminus resulted in a shift in dose-response in all cases, which indicates decreased tubulin heterodimer binding affinity. However, under the present assay conditions, each derivative was used at a concentration that was sufficient to reach the plateau level of tubulin GTPase stimulation (data not shown). Hence, the level of GTPase stimulation appears to be completely conserved among Op18/stathmin family members and requires only the region shown to be directly involved in the cooperative tubulin heterodimer binding activity, which generates the ternary tandem tubulin heterodimer complex (see Fig. 1).

Op18 has also been shown previously to inhibit tubulin GTP exchange (5, 17). A comparison of tubulin GTP exchange inhibitory properties of the three Op18/stathmin family members is shown in Fig. 2C, and the exchange rates calculated were as follows: control, 0.82 min⁻¹; Op18, 0.061 min⁻¹; sSCG10, 0.036 min⁻¹; and sRB3, 0.0076 min⁻¹. It is evident from these results that, under identical conditions to those used for tubulin GTPase assays, Op18 causes inhibition of the tubulin GTP exchange rate by a factor of ~10. Interestingly, the effects of sSCG10 and sRB3 were even more potent, and the observed GTP exchange inhibition lies in the order sRB3 > sSCG10 > Op18. This is the same order as the order reported for stability of the ternary complexes between tubulin heterodimers and Op18. This is the same order as the order reported for stability of the ternary complexes between tubulin heterodimers and Op18.

To further address the structural requirements underlying tubulin GTP exchange rates, assays were performed on ice to increase the stability of complexes. We used the same glycerol-containing buffer as in the experiments described above, because glycerol greatly increases the tubulin heterodimer binding affinity of both full-length and truncated Op18 derivatives, thereby facilitating saturated binding (19). To estimate the affinity of tubulin heterodimer binding under these conditions, plasmon resonance competition experiments were performed (Fig. 3A). The data show that truncation of the 45-residue non-helical N-terminal region results in a significant decrease in binding affinity toward tubulin heterodimers. From these data, the free tubulin concentrations at half-saturation (i.e. the apparent dissociation constant, ignoring two-site binding cooperativity) were calculated to be <0.2 μM for Op18 and 2.5 μM for Op18-R1 + 2. Analysis of the tubulin GTP exchange inhibitory properties of these two Op18 derivatives reveals similar levels of GTP exchange inhibitory activities if determined at 0 °C (Fig. 3B), and the calculated exchange rates were 0.012 min⁻¹ for Op18 and 0.015 min⁻¹ for Op18-R1 + 2 as compared with 0.16 min⁻¹ for the control. These data also show that the potency of GTP exchange inhibition is dramatically increased at 0 °C compared with 37 °C (compare Figs. 2C and 3B). However, this can be attributed in part to the fact that the GTP exchange rate of pure tubulin is reduced at 0 °C in a glycerol-containing buffer, which has also been observed by others (21).

It seems clear from the data in Fig. 3 that the non-helical
N-terminal region of Op18 is not a structural requirement for potent inhibition of GTP exchange. It follows that the enhancing effect of the N-terminal region on GTP exchange inhibition observed at 37 °C (Fig. 2) is an indirect effect mediated by the affinity-enhancing activity of this non-helical part of Op18/stathmin family members (Fig. 3A). It is also notable that the slopes of GTP exchange both at 37 °C (Fig. 2) and at 0 °C (Fig. 3) are monophasic, which suggests similar GTP exchange rates at both E-sites contained within the ternary complex. The solved x-ray structure shows that repeats 1 and 2 of Op18/stathmin family proteins are not in contact with the exposed E-site at one end of the complex, whereas the E-site that is interfaced between the two heterodimers can be predicted to be enclosed (Ref. 4; see Fig. 1). Thus, it seems that the cooperative tubulin heterodimer-binding activity of Op18-like proteins inhibits GTP exchange at the exposed E-site by an allosteric effect.

Stoichiometry of GTP Hydrolysis within the Ternary Tandem Tubulin Heterodimer Complex—To address whether GTP is hydrolyzed at one or both of the E-sites of the two head-to-tail aligned tubulin heterodimers, we performed kinetic analysis of GTP/GDP conversion within the ternary complex. However, as indicated by data in Fig. 2, the rate of GTP hydrolysis in PEM buffer is too low relative to the half-life of the complex to obtain conclusive data by this approach. Therefore, nocardazole was employed to increase the rate of hydrolysis, because this drug has been reported to enhance both basal tubulin GTPase activity (22) and the GTPase activity stimulated by Op18 (5). The addition of nocardazole results in a uniform ~5-fold increase in the GTP hydrolysis rate of tubulin heterodimers in complex with Op18, SCG10, or RB3 (data not shown).

Kinetic analysis of nocardazole enhanced [α-32P]GTP hydrolysis within ternary complexes generated using sRB3, which is the complex with the longest half-life of GTP exchange (see Fig. 2C), suggesting that only about half of the tubulin-bound GTP is susceptible to hydrolysis (Fig. 4A). Thus, in the presence of excess cold GTP, which restricts detection of hydrolysis to those complexes formed prior to the initiation of the time course, hydrolysis of labeled GTP approaches a plateau level at ~50%. This is below the level of hydrolysis observed under conditions allowing multiple rounds of [α-32P]GTP binding, i.e. in the absence of cold GTP. Interpretation of the experiment shown in Fig. 4A is somewhat complicated by the fact that GTP/GDP ratios were determined in the total assay mix, and this includes [α,32P]GTP/GDP, which may dissociate from tubulin during the time-course. To ensure that only tubulin heterodimer-associated [α,32P]GTP/GDP was monitored, the experiment was repeated under conditions in which complexes of sRB3/tubulin heterodimers were isolated at each time point by passage over a desalting column prior to analysis. Using this stringent protocol, it is clear that hydrolysis approaches 50% both in the presence and absence of excess cold GTP (Fig. 4B), which indicates that GTP is only hydrolyzed at one of the two E-sites within the ternary complex. Moreover, this experiment allows an additional conclusion, namely that GTP exchange is efficiently blocked at both E-sites of the ternary tandem tubulin heterodimer complex, even at 37 °C. Thus, preferential exchange with cold GTP at either of the two E-sites can be predicted to shift the observed plateau from 50% [α-32P]GTP, which is not observed.

Mutations within Repeat 1 of Op18 Partially Relieve Restrained Tubulin GTP Hydrolysis within the Complex—The extended helical region of Op18/stathmin family members is amphipathic, with conserved clusters of 3–4 hydrophobic residues phased to the same side of the helix (23). There are three of these hydrophobic “patches” located in repeat 1, as depicted in Fig. 5A. These patches are well conserved among all Op18/stathmin family members, with few conservative substitutions. To determine whether hydrophobic patches 1–3 are important for specific tubulin heterodimer-directed activities, mutants with Ala substitutions of hydrophobic residues were prepared (designated Op18-pmut1–3; see Fig. 5A). By employing surface plasmon resonance, the effects of these mutations on the affinity for tubulin heterodimers was determined in PEM buffer in the absence of glycerol (Fig. 5B). The data show that mutations in patch 2 alone were without detectable effect, whereas mutations in patch 1 or 3 result in significantly decreased affinity. Moreover, combined mutations in patches 2 and 3 result in a substantial reduction in the affinity for tubulin heterodimers over and above that of mutations in patch 3 alone.

The binding affinity of Op18 for tubulin heterodimers is strongly enhanced at pH values below the physiological range and shows an optimum at pH 6.5 (13). To facilitate saturated tubulin binding of Op18-pmut derivatives with decreased binding affinity, tubulin GTPase and GTP exchange assays were performed at pH 6.5 and in the presence of 25% glycerol. Analysis of GTP exchange activity, presented in Fig. 5C as the half-life of bound [α-32P]GTP, reveals various levels of decreased GTP exchange inhibitory activity. The observed differences could not be attributed to various degrees of saturation of tubulin binding, because each derivative was used at a sufficient concentration to reach a plateau level in GTP exchange inhibition (data not shown). Comparison with estimated binding affinities (Fig. 5B) shows an approximate correlation, which is consistent with the idea that the stability of the ternary complex is an important determinant of the magnitude of tubulin GTP exchange inhibition. Importantly, analysis of the initial rates of tubulin GTP hydrolysis stimulated by these mutants reveals that substitutions within patch 2 and patch 3 result in an increased hydrolysis rate, whereas mutations within patch 1 were without effect (Fig. 5D). It is also noteworthy that combined Ala substitutions at patches 2 and 3 (i.e.
Op18-pmut2/3) have an additive effect on the rate of hydrolysis. Thus, substitutions in these patches cause an increase in the conserved GTP hydrolysis rate within the tubulin heterodimer complex, and each one of the Op18-pmut derivatives shows a unique pattern of sub-phenotypes as regards the level of tubulin heterodimer-directed activities.

Patches 2 and 3 of Op18 are both in contact with the \(\alpha\)-tubulin subunit containing the interfaced E-site of the two head-to-tail aligned tubulin heterodimers (see Fig. 5) (4, 8). Because mutations at these specific hydrophobic patches alter Op18-mediated stimulation of tubulin GTPase activity, the data are consistent with the idea that GTP hydrolysis occurs at the interfaced E-site as a result of interaction with the catalytic loop located on the neighboring \(\alpha\)-tubulin subunit, as originally suggested by the structure of the complex (3). Given that the rate of GTP hydrolysis is increased after substitutions of hydrophobic residues at patches 2 and 3, it appears that these patches are important in keeping the two heterodimers in a configuration that restrains the otherwise potent GTPase productive interactions that are facilitated by the head-to-tail arrangement of the heterodimers in protofilaments.

DISCUSSION

Op18, RB3, and SCG10 are highly homologous proteins that generate similar ternary tandem tubulin heterodimer complexes that differ greatly in their stability (20). Because excess cold GTP shows no effect on the rate or magnitude of \(\alpha\)-GTP hydrolysis within the extraordinarily stable RB3-tubulin heterodimer complex (Fig. 4B), the present analysis indicates that exchange inhibition is essentially complete at both of the two E-sites within the complex. One of these E-sites is interfaced between the two head-to-tail-aligned tubulin heterodimers, and it can be envisioned that GTP at this interfaced E-site is well enclosed and protected from exchange (see model in Fig. 5A). However, the other E-site is completely exposed, and it follows that tubulin GTP exchange inhibition at this E-site must be explained by an allosteric effect.

It is evident from the present analysis that differences in the
stability of ternary tandem tubulin heterodimer complexes are also manifested as major differences in the magnitude of GTP exchange inhibition at 37 °C (Fig. 2, C and D). Moreover, analysis of GTP exchange inhibition by Op18 derivatives with destabilizing Ala substitutions in hydrophobic patches also suggests that the stability of the ternary tandem tubulin heterodimer complex is of primary importance for the potency of GTP exchange inhibition (Fig. 5C). These findings suggest that complex dissociation is required for GTP exchange to occur. We have previously reported that GTP exchange inhibition by Op18 at 37 °C is partially dependent on the non-helical N-terminal region (5), and the present studies extend this finding to the sRB3 and sCCG10 proteins (Fig. 2, C and D).

Binding analysis shows that the non-helical N-terminal region of Op18 contributes to the affinity of tubulin heterodimer binding (Fig. 3A), and it follows that the observed importance of this region for GTP exchange inhibition can readily be explained as an indirect consequence of increased complex stability. Consistent with this interpretation, it is clear from analysis of GTP exchange inhibition at 0 °C that the extended helix of Op18 is sufficient for optimal inhibition of GTP exchange at both sites of the complex (Fig. 3B).

The present determination of the stoichiometry of GTP hydrolysis within the ternary tandem tubulin heterodimer complex, combined with the phenotype of Ala substitutions at distinct hydrophobic clusters along the face of the extended helix of Op18, is most consistent with GTP hydrolysis occurring at the interface E-site only (see Fig. 5A). The most likely mechanism involves interactions between the interfaced E-site and the catalytic loop located on the neighboring α-tubulin subunit, as originally suggested by the head-to-tail arrangement of heterodimers in the complex (3). This simple model is also consistent with the finding that the rate of tubulin GTP hydrolysis within the complex is independent of the non-helical N-terminal region of Op18/stathmin family members (Fig. 2). It is notable that the conserved rate of GTP hydrolysis within a ternary tandem tubulin heterodimer complex is several orders of magnitude lower than during polymerization-facilitated interactions between the E-site and the catalytic loop of an adjacent heterodimer. This indicates that Op18/stathmin family members keep the two heterodimers in a configuration that restrains the otherwise potent GTPase productive interactions facilitated by the head-to-head alignment of heterodimers in protofilaments (10). In the present study, we have identified structural hydrophobic motifs that are at least in part responsible for restraining GTPase productive interactions, as evidenced by the observed phenotypes of Ala substitutions in the conserved hydrophobic patches 2 and 3 of Op18 (Fig. 5). It is of particular interest that substitutions at patch 2 resulted in increased GTP hydrolysis within the ternary tandem tubulin heterodimer complex, with minimal effects on tubulin heterodimer binding affinity and GTP exchange inhibition. This indicates the specificity and importance of these hydrophobic patches for restraining GTPase productive interactions between the two head-to-tail-aligned tubulin heterodimers within the ternary complex.

The present mutational analysis of conserved hydrophobic patches reveals a unique pattern of sub-phenotypes regarding the level of tubulin heterodimer binding, GTP exchange, and hydrolysis. Although there is a correlation between tubulin heterodimer binding affinity and GTP exchange inhibition, our data uncouple tubulin heterodimer binding affinities from the stimulation of GTP hydrolysis. However, in order to generate GTPase productive ternary complexes, it is essential that binding should occur with sufficient positive cooperativity such that the formation of hydrolysis-incompetent binary complexes containing a single tubulin heterodimer would be minimized. In the absence of glycerol, the Op18-pmut1 derivative shows a positive binding cooperativity that is too low to efficiently form GTPase productive tandem tubulin heterodimer complexes (data not shown). This is also manifested as a low tubulin heterodimer binding affinity in the present plasmon resonance competition analysis, which was performed in a glycerol-free buffer (Fig. 5D). Consistent with decreased positive binding cooperativity under these conditions, Op18-pmut1 is inefficient in stimulating tubulin GTP hydrolysis if the assay is performed in the absence of glycerol (19). However, the addition of glycerol to the buffer increases positive binding cooperativity (data not shown), and allows efficient generation of ternary tandem tubulin heterodimer complexes with normal levels of GTP hydrolysis (Fig. 5D). This illustrates that glycerol, by altering cooperative tubulin heterodimer binding properties, may also alter specific phenotypes of mutant derivatives, which may in some cases confuse interpretation of in vitro results. In the present study, glycerol was included in the different assays to allow comparison of truncated/mutated derivatives with decreased tubulin heterodimer binding affinity, generating conditions in which binding approached saturation in all cases. It should be noted, however, that the Op18-pmut2 and 3 derivatives showed increased tubulin GTPase stimulatory activities both in the absence and presence of glycerol (data not show). Moreover, although decreased binding cooperativity may explain an apparent decrease in the stimulation of tubulin GTP hydrolysis as outlined above, the observed increase in stimulation of GTP hydrolysis caused by mutations of Op18 patches 2 and 3 cannot be explained by alterations in the level of binding cooperativity. That Op18 modulates the GTP exchange and hydrolysis properties of tubulin heterodimers has evoked speculation previously on the potential significance of these activities for the mechanism underlying catastrophe promotion (5). However, catastrophe promotion, which requires the non-helical N-terminal region of Op18 (14, 24), does not depend on the formation of GTPase productive/GTP exchange-inhibited ternary complexes. Hence, the present analysis, which was prompted by recent structural insights into the architecture of the ternary complex (3, 4), indicates a conserved and very different role for these GTP exchange/hydrolysis modulatory activities, namely to prevent futile cycles of GTP hydrolysis.

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