Deciphering the Cellular Functions of the Op18/Stathmin Family of Microtubule-Regulators by Plasma Membrane-targeted Localization

Per Holmfeldt, Kristoffer Brännström, Sonja Stenmark, and
Martin Gullberg*

Department of Molecular Biology University of Umeå, SE-901 87 Umeå, Sweden

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The Op18/stathmin family of microtubule regulators includes the ubiquitous cytosolic Op18/stathmin (Op18) and the neuronal, primarily Golgi-associated proteins SCG10 and RB3, which all form ternary complexes with two head-to-tail-aligned tubulin heterodimers. To understand the physiological significance of previously observed differences in ternary complex stability, we have fused each of the heterodimer-binding regions of these three proteins with the CD2 cell surface protein to generate confined plasma membrane localization of the resulting CD2 chimeras. Herein, we show that, in contrast to constitutively active CD2-Op18-tetraA, both the CD2-SCG10 and CD2-RB3 chimeras sequestered tubulin at the plasma membrane, which results in >35% reduction of cytosolic tubulin heterodimer levels and consequent delayed formation of mitotic spindles. However, all three CD2 chimeras, including the tubulin sequestration-incompetent CD2-Op18-tetraA, destabilize interphase microtubules. Given that microtubules are in extensive contact with the plasma membrane during interphase, but not during mitosis, these findings indicate that Op18-like proteins have the potential to destabilize microtubules by both sequestration and direct interaction with microtubules. However, the differences in tubulin binding observed in cells also indicate conceptual differences between the functions of low-abundance neural family members, which will accumulate tubulin at specific cellular compartments, and the abundant cytosolic Op18 protein, which will not.

INTRODUCTION

The Op18/stathmin family of microtubule regulators includes the ubiquitous cytosolic Op18/stathmin (Op18) and three closely related neural-specific and neural growth-associated gene products termed RB3, SCG10, and SCLIP (for review, see Mori and Morii, 2002). These homologous proteins form a complex with two tubulin heterodimers arranged head-to-tail (Gigant *et al.*, 2000; Steinmetz *et al.*, 2000); however, the stability of this ternary complex varies extensively among Op18-like proteins (Charbaut *et al.*, 2001). A characteristic feature of the neural family members is a hydrophobic N terminus, which includes two Cys residues that serve as palmitoylation sites. This N terminus mediates association to intracellular membranes, primarily at the

Golgi apparatus but also within growth cones (Di Paolo *et al.*, 1997; Gavet *et al.*, 1998; Lutjens *et al.*, 2000). On the other hand, the ubiquitous Op18 protein lacks the hydrophobic part of the N terminus and has a widespread cytosolic distribution.

Microtubules (MTs) are highly dynamic polymers that switch stochastically between growing and shrinking phases by catastrophe and rescue events (for review, see Desai and Mitchison, 1997). The dynamic behavior of MTs is regulated both in vitro and in intact cells by Op18/stathmin family members (for review, see Cassimeris, 2002). Op18 is phosphorylated at four Ser residues by both cell cycle-regulating and signal-transducing kinase systems (for review, see Lawler, 1998). Modifications at these sites result in various degrees of functional inactivation of Op18 (Marklund *et al.*, 1996; Horwitz *et al.*, 1997; Larsson *et al.*, 1997). Multisite phosphorylation of Op18 at the start of mitosis is required for spindle formation (Larsson *et al.*, 1995), whereas phosphorylation of Op18 by signal-transducing kinases has been shown to mediate regulation of the interphase array of MTs (Melander Gradin *et al.*, 1997; Gradin *et al.*, 1998). All four Ser phosphorylation sites of Op18 are conserved in SCG10 and

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* Corresponding author. E-mail address: martin.gullberg@molbiol.umu.se.

Abbreviations used: MAP4, microtubule-associated protein 4; MT, microtubule; Op18, oncoprotein 18/stathmin.

SLIP, which may be of significance for regulation of the MT system during neural differentiation (Antonsson *et al.*, 1998; Ozon *et al.*, 1998). However, RB3 lacks three of the four sites and the remaining site, which corresponds to Ser-16 of Op18, has a nonconserved consensus sequence (Ozon *et al.*, 1997).

Op18-like proteins can be divided into two distinct structural regions, namely, a nonhelical 45-residue N-terminal region and an extended C-terminal helix containing two imperfect repeats, each of which makes contact with one tubulin heterodimer (Figure 1A; Gigant *et al.*, 2000; Wallon *et al.*, 2000). Op18 was originally shown to destabilize MTs by a catastrophe-promotion mechanism, i.e., transition from a growing to a shrinking MT (Belmont and Mitchison, 1996), and subsequent molecular dissection has suggested that an intact N terminus is both sufficient and necessary for this type of destabilizing activity (Howell *et al.*, 1999b; Segerman *et al.*, 2003). Op18 has also been shown to mediate tubulin sequestration (Curmi *et al.*, 1997; Jourdain *et al.*, 1997), which is independent of the N terminus but requires the two imperfect repeats of the extended helix (Howell *et al.*, 1999b) that are responsible for high-affinity, two-site positively cooperative binding of two tubulin heterodimers (Gigant *et al.*, 2000).

The activity profiles of truncated Op18 derivatives expressed or microinjected into intact cells has supported the notion that catastrophe promotion and tubulin sequestering are independent activities (Larsson *et al.*, 1999; Holmfeldt *et al.*, 2001). This concept of functional dichotomy has gained independent support from a study in which we determined how ectopic expression of the MT-stabilizing MAP4 protein counteracts distinct MT-destabilizing proteins (Holmfeldt *et al.*, 2002). Thus, it was demonstrated that MAP4 only counteracts catastrophe-promoting proteins that lack tubulin-sequestering activity, which allowed us to confirm the presence or absence of sequestering activity in previously characterized Op18 truncation derivatives in intact cells.

Although molecular dissection of Op18 has indicated bifunctional properties, the relative importance of catastrophe promotion versus tubulin-sequestering activities in intact cells remains unknown. Herein, we have approached this question by targeting different Op18/stathmin family members to a confined cellular location. This has allowed a direct correlation between reduced free tubulin concentrations and MT-destabilizing activities mediated by Op18-like proteins.

MATERIALS AND METHODS

Transfection and Cell Culture

pMEP4 shuttle vector derivatives expressing full-length Op18-tetraA-FLAG, Op18(1-99)-tetraA, Op18(25-149)-triA, and MAP4 (isoform III; Chapin *et al.*, 1995) have been described previously (Holmfeldt *et al.*, 2002; Larsson *et al.*, 1999). Op18 truncation derivatives are denoted by numbers within brackets, indicating the amino acid residues present, and -tetraA refers to substitution of the Ser-16, Ser-25, Ser-38, and Ser-63 phosphorylation sites with Ala residues. The RB3 (residues 45–189)-FLAG and SCG10 (residues 35–179)-FLAG derivatives, which corresponds to the Op18-like region, were prepared by a polymerase chain reaction (PCR) strategy as described previously (Brannstrom *et al.*, 2003). The CD2 chimeras were prepared by a PCR strategy using a full-length cDNA clone of rat CD2 (accession number X05111; kindly provided by Dr. Allan Williams; He *et al.*, 1988) as template with primers 5'-GCG GCG CCA

TGG CGA GAT GTA AAT TCC TAG GG-3' and 5'-CGC GCG CCA TGG CGC ACG CGT TCC GTT TTT TCC TCT TGC-3'. The resulting PCR fragment contained *NcoI* sites at both ends and a coding sequence corresponding to aa 1–212 of rat CD2. The *NcoI*-digested fragment was inserted into the corresponding site of RB3 (residues 45–189)-FLAG, SCG10 (residues 35–179)-FLAG, Op18-tetraA-FLAG, or Op18 (residues 56–149)-FLAG. The coding sequence of the PCR-generated fragments was confirmed by nucleotide sequence analysis by using the ABI PRISM dye terminator cycle sequencing kit from PerkinElmer Life Sciences (Boston, MA). For expression in cell lines, coding regions for each CD2 chimera were subcloned as *HindIII* to *BamHI* fragments into the Epstein-Barr virus-based shuttle vector pMEP4 (Invitrogen, Carlsbad, CA). Conditional expression/coexpression was induced from the hMTIIa promoter, which can be suppressed by cultivation in a specifically formulated medium and subsequently induced by 0.5 μM Cd^{2+} (Marklund *et al.*, 1996). Single transfection and cotransfection by using pMEP4 derivatives, and subsequent selection of hygromycin-resistant cell lines >4–6 d, were performed as described previously (Gradin *et al.*, 1998) by using a total of 18 μg of DNA. For coexpression of MAP4 and Op18-like derivatives, we used 6 μg of pMEP-MAP4 DNA in a mix with a total of 18 μg of pMEP-derivative DNA.

Immunoblotting and Immunofluorescence

Immunoblotting and subsequent detection using the enhanced chemiluminescence detection system (Amersham Biosciences) were performed using anti-MAP4 (M75820; Transduction Laboratories, Lexington, KY), anti- α -tubulin (B-5-1-2; Sigma-Aldrich, St. Louis, MO), and anti-rat CD2 (MRC OX-34; He *et al.*, 1988). To allow simultaneous and equivalent detection of all Op18/stathmin family members, rabbit antibodies were raised against a peptide corresponding to a completely conserved region located between residues 46–58 of Op18 (SLEEIQKKLEAAE). The resulting antibodies were affinity purified by absorption to native Op18 coupled to Sepharose. Analysis of cellular MT content by flow cytometry (>95% of all cells were included in the acquisition gate and >200,000 cells were collected) was performed using an FACS Calibur instrument (Becton Dickinson, Fullerton, CA) as described previously (Holmfeldt *et al.*, 2001) with some recent modifications (Holmfeldt *et al.*, 2002). Gating of cells to determine MT-polymer mass specifically in interphase (G1/S) cells was performed by the criterion of DNA content, as detected by propidium iodine staining of DNA. In experiments involving cells transfected with plasma membrane-associated CD2 chimeras, saponin in the MT-stabilizing buffer was replaced with 0.2% Triton X-100. This revision of the protocol results in complete solubilization of CD2 chimeras. Quantification of MAP4, α -tubulin, and Op18-like proteins by flow cytometry was performed on cells chilled on ice, to depolymerize MTs, followed by fixation by using 2% paraformaldehyde. Fixed cells were permeabilized with saponin (0.2%), and staining was performed with the appropriate primary and secondary antibodies as described previously (Holmfeldt *et al.*, 2002). For characterization of spindles by immunofluorescence analysis, cells were permeabilized with saponin (0.2%) in MT-stabilizing buffer and subsequently fixed in 4% paraformaldehyde/0.5% glutaraldehyde, followed by quenching with NaBH_4 . MTs and DNA were costained using Alexa Fluor 488-conjugated anti- α -tubulin and propidium iodide and analyzed by epifluorescence. To evaluate tubulin accumulation and consequent reduction of cytosolic free tubulin in cells expressing the CD2 chimera, cells were directly fixed in 4% paraformaldehyde at 37°C. Fixed cells were permeabilized with saponin (0.2%) and CD2 chimeras and tubulin were costained with biotinylated anti-CD2/R-phycoerythrin-conjugated streptavidin and Alexa Fluor 488-conjugated anti- α -tubulin, respectively, and analyzed using a Leica SP2 confocal imager system (Marklund *et al.*, 1996). To estimate cytosolic levels of free tubulin in mitotic cells, fluorescence intensities of Alexa Fluor 488 within 10 randomly chosen circular MT-free areas (radius $\sim 0.25 \mu\text{m}$) in the cytosol were evaluated in a confocal

Figure 1. Effect of pH and physiological ionic strength on tubulin heterodimer complex formation by Op18-like proteins. (A) Schematic representation of Op18 depicted with a nonhelical N-terminal region (residues 1–45) and an extended α -helical region containing two low-homology repeats, designated repeats 1 and 2, which are separated by 51 residues (Gigant *et al.*, 2000). The positions of two longitudinally arranged tubulin heterodimers, depicted as spheres, are based on the low-resolution x-ray structure (Gigant *et al.*, 2000). The flag and oval indicate the C-terminal FLAG epitope tag and Cys-rich fusion partner present on proteins used in plasmon resonance studies. Equivalent fused derivatives of SCG10 and RB3 are truncated derivatives that lack the native hydrophobic N terminus and are thus soluble proteins that contain only the cognate Op18-like region and the C-terminal fusions (see MATERIALS AND METHODS). (B–D) Surface plasmon resonance sensorgram of full-length Op18 (B) and the Op18-like regions of SCG10 (C) and RB3 (D) coupled via the C-terminal Cys-rich sequence fusion partner. Association kinetics were analyzed in the presence of 4 μ M tubulin at 25°C in PEM, pH 6.8 (solid lines), PEM, pH 7.4 (dotted lines), or PEM, pH 7.4, containing 120 mM KCl (dashed lines), as indicated. Dissociation kinetics was analyzed by a flow of the indicated buffer alone. (E) Plasmon resonance competition experiments at 25°C using PEM buffer, pH 7.4, containing 120 mM KCl. Graded concentrations of the Op18, RB3, and SCG10 were mixed with 4 μ M tubulin heterodimers and run over a flow cell coupled with SCG10 to monitor the free tubulin concentration. The dotted line depicts complete complex formation at a 1:2 M ratio of Op18-like proteins/tubulin heterodimers. The apparent dissociation constants of each Op18-like protein, which are indicated in the figure, were derived from binding curves in which the level of complex formation was plotted against the free tubulin concentration. The molar concentrations of individual preparations of Op18-like proteins were adjusted to fit in with the established 1:2 M ratio of Op18-like proteins/tubulin heterodimers.

section. To ascertain that only tubulin-specific fluorescence was quantified, the analysis was performed on cells stained with Alexa Fluor 488-conjugated anti- α -tubulin alone. Using the same general approach, plasma membrane-associated tubulin was analyzed from the same confocal section within ten randomly chosen rectangular areas of the plasma membrane. Special care was taken not to bleach the fluorescence signal before confocal scanning.

Determination of Tubulin Heterodimer Binding by Plasmon Resonance Experiments

Directional thiol-specific coupling of Op18 and the Op18-like regions of RB3 (residues 45–189) and SCG10 (residues 35–179) to BIAcore sensor chips was achieved via a C-terminally fused Cys-rich sequence derived from the zebra fish metallothionein (residue 2–60, 34% Cys residues, accession number NM_131075; kindly provided by Dr. Per Erik Olsson, University of Umea, Umea, Sweden). The metallothionein sequence was fused proximal to a C-terminal 8-residue FLAG epitope tag of Op18, SCG10, and RB3 derivatives by using a PCR strategy (primer sequences are available from the authors on request). The resulting fusion proteins were expressed with a six-residue His-tag at the N terminus and purified from *Escherichia coli* by using pET-3d expression as described previously (Larsson *et al.*, 1999). Plasmon resonance binding experiments were carried out on a BIAcore 3000 system with the C-terminally fused Cys-rich sequence immobilized by the ligand thiol method on a CM5 chip according to the instructions of the manufacturer. Glycine (5 mM) was present during the coupling reaction to minimize immobilization via the free amine groups of Op18-like proteins. All plasmon resonance binding experiments were run using PEM buffer (80 mM piperazine-*N,N'*-bis[2-ethanesulfonic acid], 1 mM EGTA, 4 mM Mg^{2+}) adjusted to the indicated pH with NaOH. Where indicated, this buffer also contained 120 mM KCl or 10% glycerol. Plasmon resonance competition experiments were carried out with immobilized SCG10. Analyses were performed with the indicated concentrations of tubulin in PEM buffer, pH 7.4, containing 120 mM KCl, premixed with graded concentrations of Op18-like derivatives. The free tubulin concentrations were determined from the plateau levels by comparison with a standard tubulin curve, as described in the BIAcore handbook. Calculation of apparent dissociation constants (K_d) was made with the assumption of a 1:2 M ratio of Op18-like proteins/tubulin heterodimers (Gigant *et al.*, 2000).

RESULTS

Tubulin Affinities of Op18/Stathmin Family Members in Various Buffer Systems

The two neural Op18/stathmin family members SCG10 and RB3 have stretches of hydrophobic residues at their N terminus that mediate membrane targeting. DNA encoding these hydrophobic stretches was deleted to produce soluble truncated proteins corresponding to the cognate Op18-like region. Analysis of tubulin heterodimer binding by these Op18-like proteins was performed using a strategy that involved coupling via a Cys-rich fusion partner to BIAcore chips (Figure 1A). Given that this coupling procedure does not affect the tubulin-binding regions, the tubulin-binding properties of Op18-like proteins can be directly compared. As shown in Figure 1, B–D, determination of interaction kinetics by plasmon resonance under various buffer conditions showed that Op18, SCG10, and RB3 have very different tubulin-binding properties. The observed differences, including the observation that RB3 has a slow on and off kinetic, are consistent with the result of a previous study in

which binding properties of Op18-like proteins were analyzed in PEM buffer, pH 6.8 (Charbaut *et al.*, 2001). Most importantly, it is also clear from the data that the pH and ionic strength is of major importance for the tubulin binding properties of each of the Op18-like proteins.

The tubulin-binding affinity of Op18 is increased approximately fivefold by the addition of glycerol to the buffer (Larsson *et al.*, 1999). Accordingly, to compare the tubulin-binding capacity of Op18-like proteins under conditions of maximal affinities, we analyzed binding in PEM buffer at pH 6.8 containing 10% glycerol. Under these conditions, all three Op18-like proteins were found to bind comparable amounts of tubulin with plateau levels of response units (RUs) being 1002 RUs for Op18, 1415 RUs for SCG10, and 1442 RUs for RB3. However, from the sensorgrams shown in Figure 1, B–D, which depict tubulin binding in glycerol-free buffers, it becomes clear that both SCG10 and RB3 generally bind severalfold more tubulin heterodimers and form more stable complexes than Op18. Importantly, the differences in tubulin heterodimer binding properties between Op18 and the two neural Op18-like proteins becomes even more pronounced if binding is analyzed at increased pH, and at physiological KCl concentration. It is also evident that SCG10 is less sensitive to altered pH and ionic strength conditions than Op18 and RB3. Thus, under conditions that are close to physiological in terms of pH and ionic strength (i.e., pH 7.4 and 120 mM KCl), SCG10 seems to have somewhat higher apparent tubulin-binding affinity than RB3, and the affinity of Op18 seems to be low.

To compare complex stability, we determined dissociation rates, which are expressed in Table 1 as the half-life of complex dissociation. The data revealed that the presence of glycerol in the binding buffer exerts a stabilizing effect on the tubulin heterodimer complexes formed by all three Op18-like proteins, but the relative degree of stabilization by glycerol varied in the order Op18 > RB3 > SCG10. It is also clear that the stability of the complexes is dramatically decreased in all cases in a buffer system designed to be similar to cytosolic conditions in terms of pH and ionic strength. A temperature rise from 25°C to 37°C further reduced the tubulin complex stability of all three Op18-like proteins, and at the physiological temperature the dissociation kinetic for Op18 was too fast to be determined. Thus, under conditions aimed at being similar to physiological conditions, all three Op18-like proteins form much more unstable ternary tubulin complexes, with the Op18 ternary complexes being particularly unstable.

To determine the tubulin-binding dissociation constants of Op18-like proteins in solution, plasmon resonance competition experiments were performed in PEM buffer at pH 7.4 containing 120 mM KCl. As shown in Figure 1E, the apparent dissociation constants, i.e., the free tubulin concentration at half saturation, lies in the order Op18 > RB3 > SCG10. Given that the cytosolic levels of nonpolymerized tubulin heterodimers are likely to be in the 5–10 μ M range, it seems clear that the two neural Op18-like proteins have sufficient affinities to undergo complex formation with cellular tubulin that is close to stoichiometric. However, the modest affinity of Op18 under physiological conditions predicts a lower level of tubulin complex formation.

Table 1. Summary of dissociation rates of tubulin bound to Op18-like protein derivatives under various conditions

Temperature	Condition	Op18 $t_{1/2}$ (s)	SCG10 $t_{1/2}$ (s)	RB3 $t_{1/2}$ (s)
25°C	pH 6.8 + 10% Gly	77.5 ± 2.5	132.5 ± 7.5	1930.5 ± 29.5
	pH 6.8	15.5 ± 0.5	93.25 ± 0.25	654 ± 4
	pH 7.4	3.25 ± 0.25	54.5 ± 3.5	259 ± 15
	pH 7.4 + 120 mM KCl	1.6 ± 0.1	35 ± 1	163.9 ± 0.9
37°C	pH 6.8 + 10% Gly	21.5 ± 0.5	78.5 ± 3.5	990 ± 10
	pH 6.8	3 ± 0.5	20 ± 2	305 ± 5
	pH 7.4	0.55 ± 0.15	7.75 ± 0.25	76 ± 2
	pH 7.4 + 120 mM KCl	<0.25	3.75 ± 0.25	32 ± 1

The data are derived from plasmon resonance analysis of dissociation kinetic curves under the indicated condition. $T_{1/2}$ represents apparent tubulin dissociation rates, i.e. the time for 50% dissociation. The data presented represent the mean $T_{1/2}$ (+/- standard error) after prebinding of 2- and 4- μ M tubulin to chips coupled with the indicated Op18-like proteins.

Tubulin Heterodimer Binding Properties of Plasma Membrane-localized Op18/Stathmin Family Members in Permeabilized and Intact Cells

To analyze tubulin binding by distinct Op18-like proteins in intact cells, we created chimeric protein derivatives that are targeted and thus localized to the plasma membranes (Figure 2A). This was achieved by fusion of Op18 and the Op18-like regions of SCG10 and RB3 with the extracellular and transmembrane region of the T lymphocyte-specific cell surface protein CD2. It should be noted that the CD2-Op18-tetraA chimera is nonphosphorylatable, because it has Ala substitutions of the four Ser residues at all four phosphorylation sites and thus cannot be inactivated by phosphorylation (Larsson *et al.*, 1997; Marklund *et al.*, 1996). This is also the case for the CD2-RB3 chimera, but not the CD2-SCG10 chimera, because in contrast to SCG10 the native RB3 protein lacks three important serine phosphorylation sites and the remaining site has a nonconserved consensus sequence. As a control, a CD2 chimera was prepared that contained a 95-residue C-terminal part of Op18 that has previously been shown to be unable to bind tubulin heterodimers (CD2-Co) (Larsson *et al.*, 1999). These CD2 derivatives were expressed in the human K562 leukemia cell line by using the replicating pMEP4 vector system, which allows rapid establishment of homogeneous transfected cells lines and stringent regulation of ectopic expression from the hMTIIa promoter (Marklund *et al.*, 1994). Using this system, addition of Cd^{2+} resulted in transient expression with a peak within 5–6 h (Melander Gradin *et al.*, 1997). Immunoblot analysis of expressed CD2 chimeras shortly after Cd^{2+} stimulation mostly revealed the immature nonglycosylated proteins (our unpublished data). Hence, to allow sufficient time for the expressed CD2 chimeras to travel via the exocytic pathway to the plasma membrane, transfected K562 leukemia cells were Cd^{2+} induced for 20 h. After 20 h of induction, immunoblots probed with anti-CD2 (Figure 2B) revealed uniform expression levels of all four CD2 chimeras, which all migrate as broad heterogeneous bands characteristic of mature glycosylated membrane proteins. The same samples were also analyzed by probing immunoblots with antibodies against a peptide corresponding to a completely conserved region among Op18-like proteins (anti-SLEEIQ) (Figure 2B). Arbi-

trary quantification of the signal generated by each of the CD2 chimeras relative to endogenous Op18 was obtained from serial dilutions of cell lysates (our unpublished data). Integration of the heterogeneous band signal indicated that CD2 chimeras are expressed at an approximately eightfold higher level than endogenous Op18.

Permeabilization of transfected cells with saponin does not result in significant solubilization of the plasma membrane-associated CD2 chimeras, whereas as expected, Triton X-100 caused essentially complete solubilization (Figure 2C). Given that CD2 chimeras are retained after permeabilization with saponin, binding of endogenous tubulin heterodimers by CD2 chimeras could be compared after fixation of cells by determining tubulin content by flow cytometry. Accordingly, cells were diluted in a permeabilizing saponin/PEM/KCl buffer, designed to mimic cytosolic condition in terms of pH, Mg^{2+} , and ion strength (Figure 2D). To depolymerize all MTs, prechilled cells were kept on ice during the 7-min incubation with permeabilization buffer. The result revealed that CD2-Op18-tetraA does not retain endogenous tubulin under these conditions, whereas CD2-SCG10 and CD2-RB3 retained a significant fraction of all endogenous tubulin heterodimers. However, by using nonphysiological buffer conditions that were previously shown to strongly enhance tubulin heterodimer affinity (i.e., PEM, pH 6.8, and 10% glycerol, Table 1; Larsson *et al.*, 1999), it is clear that the CD2-Op18-tetraA is also capable of binding a significant fraction of all endogenous tubulin heterodimers and is thus functional.

To estimate the potential of expressed CD2 chimeras to accumulate tubulin heterodimers at the plasma membrane, transfected cells were prechilled on ice to depolymerize all MTs, and fixed thereafter. Confocal sections show that the CD2 chimeras are all expressed at comparable levels at the plasma membrane, as detected by an anti-CD2 antibody (Figure 3A, top). It is also evident that CD2-SCG10 and CD2-RB3, but not CD2-Co and CD2-Op18-tetraA, cause plasma membrane accumulation of tubulin (Figure 3A, bottom). To quantify the observed accumulation, the average fluorescence intensity of cytosolic and membrane-associated tubulin was determined within a confocal section (see MATERIALS AND METHODS). The data indicated that al-

Figure 2. Conditional expression of chimeras between the transmembrane receptor CD2 and Op18/stathmin family members. (A) Schematic representation of the transmembrane CD2 chimeras, which localize the tubulin heterodimer-binding region of Op18-like proteins

though CD2-Co and CD2-Op18-tetraA had no effect, both CD2-SCG10 and CD2-RB3 caused almost a 50% reduction in the diffuse staining of tubulin heterodimers in cells with their entire MT system depolymerized by cold treatment (Figure 3B). The significance of the present quantification is indicated by a negative correlation between the fluorescence intensities at the plasma membrane and the cytosol among CD2-SCG10- and CD2-RB3-expressing cells, whereas the CD2-Co and CD2-Op18-tetraA-expressing cells show the expected positive correlation (Figure 3C).

To determine the tubulin-binding activity of expressed CD2 chimeras under physiological conditions, transfected cells were directly fixed at 37°C. It is evident from Figure 3A that the spherical K562 cells have a very small cytoplasm. During interphase, the MT-array occupies most of the small cytosolic space and is in extensive contact with the entire plasma membrane. Because this obstructs the determination of free cytosolic and plasma membrane-associated tubulin, analysis was restricted to mitotic cells that had most of their MTs concentrated around the spindle (the distributions of MTs in interphase and mitotic K562 cells can be seen in Figure 8). The analysis was restricted to CD2-Op18-tetraA and CD2-RB3, because both of these derivatives, in contrast to CD2-SCG10, are resistant to inactivation by phosphorylation during mitosis, as described above. The data in Figure 4 obtained at 37°C are in agreement with data from cold-treated cells, because CD2-RB3 clearly accumulates tubulin to the plasma membrane, whereas CD2-Op18-tetraA does not.

The results described above are consistent with the idea that the neural SCG10 and RB3 proteins have much higher heterodimer-binding affinities than the ubiquitous Op18 protein. Given previous evidence that overexpressed Op18 derivatives mediate tubulin-sequestering activity in intact cells (Holmfeldt *et al.*, 2002), it was initially surprising that the CD2-Op18-tetraA chimera did not cause detectable tubulin accumulation at the plasma membrane. However, efficient capture at a confined location requires higher affinity than is required for formation of soluble cytosolic seques-

Figure 2 (cont). and thus tubulin heterodimers (represented as spheres) to the plasma membrane. The complete Op18-tetraA sequence was fused at its N terminus to the intracellular part of rat CD2. The regions of RB3 and SCG10 fused to CD2 correspond to the complete Op18 sequence by analogy with the cognate derivatives presented in Figure 1A. (B) Transfected K562 cell lines harboring the indicated episomally replicating pMEP-CD2 chimera were analyzed after 20 h of Cd²⁺-induced expression from the hMTIIa promoter. Immunoblots of total cellular lysate (20 µg/lane), separated by 12% SDS-PAGE, were performed using the indicated antibodies for detection. Anti-SLEEIQ recognizes all Op18-like proteins. The position of endogenous Op18 is indicated by an arrow at 18.5 kDa. (C) Prechilled transfected K562 cells were kept on ice for 7 min in an MT-stabilizing PEM buffer designed to mimic cytosolic conditions (pH 7.4 and 120 mM KCl), either under nonpermeabilizing conditions (black bars) or in the presence of saponin (shaded bars) or Triton X-100 (open bars). After fixation, the amount of cell-associated CD2 chimeras was determined by staining with anti-CD2 followed by flow cytometry. (D) Cells were permeabilized on ice as in C, in saponin containing MT-stabilizing buffers (pH 7.4 or 6.8) supplemented with either 120 mM KCl or 10% glycerol, as indicated. The cells were subsequently fixed and the amount of cell-associated tubulin was determined by staining with anti-α-tubulin followed by flow cytometry. The data are representative of three independent transfection experiments.

Figure 3. Estimation of cytosolic free tubulin concentrations in CD2 chimera-expressing cells after cold-induced depolymerization of MTs. Transfected K562 cell lines harboring the indicated pMEP-CD2 chimera derivative were Cd^{2+} -induced for 20 h and put on ice for 30 min to depolymerize the MT system. The cells were subsequently fixed and costained with anti-CD2 (R-phycoerythrin) and anti- α -tubulin (Alexa Fluor 488). (A) Confocal sections of representative CD2 chimera-expressing interphase cells. Bars, 6 μm . (B) Average fluorescence intensity of nonpolymeric α -tubulin that was determined in the cytosol and at the plasma membrane in confocal sections of cells expressing the indicated CD2 chimera. The fluorescence intensities of individual cells expressing the CD2-Co ($n = 22$), CD2-Op18-tetraA ($n = 19$), CD2-SCG10 ($n = 24$), and CD2-RB3 ($n = 28$) are plotted in C. All data in this figure are derived from the same transfected cell populations but are representative of at least three independent transfection experiments.

tering-complexes, because the heterodimer affinity has to be sufficient to maintain the resulting concentration gradient within the cell. In the case of the CD2-SCG10 and CD2-RB3 chimeras, it is apparent that the intrinsically higher binding affinity is sufficient for accumulation of a large pool of cytosolic tubulin at the plasma membrane.

MT Destabilization by Plasma Membrane-localized Op18/Stathmin Family Members in Intact Cells

The potency by which the CD2 chimeras destabilize cellular MTs was analyzed by determination of the fraction of polymerized tubulin in transfected cells. The result demonstrates that CD2-Op18-tetraA was almost as efficient in destabilizing the interphase MT-array as the CD2-SCG10 and CD2-RB3 chimeras (Figure 5). The observed MT destabilization caused by the latter two chimeras is readily explained by

tubulin accumulation at the plasma membrane and consequent reduction of cytosolic free tubulin concentrations. However, the CD2-Op18-tetraA chimera did not capture detectable amounts of tubulin, which suggest that MT destabilization may also occur by an alternative mechanism. For example, given that the interphase MT-array is in extensive contact with the inner surface of the cell, MTs may be destabilized by a catastrophe-promoting mechanism that involves direct physical contact with plasma membrane-targeted CD2-Op18-tetraA protein.

To address the mechanism(s) by which CD2 chimeras destabilize MTs, we exploited our previous observation that MAP4 counteracts destabilization of interphase MTs caused by catastrophe promotion, but not by tubulin-sequestering activity (Holmfeldt *et al.*, 2002). Thus, if ectopic MAP4 does not counteract MT destabilization, this would indicate sequestering activity. The experimental strategy for evaluating

Figure 4. Estimation of cytosolic free tubulin concentrations in mitotic cells expressing CD2 chimeras. Transfected K562 cell lines were Cd^{2+} -induced as described under Figure 3, but kept at 37°C and fixed under conditions that preserve the MT system. Fixed cells were stained as in Figure 3 and the average fluorescence intensity of nonpolymeric α -tubulin in confocal sections was determined in the cytosol and at the plasma membrane in mitotic cells, in which MTs have a well-defined localization within the spindle. The average values of cells expressing the indicated CD2 chimera are presented in A. The fluorescence intensities of individual cells expressing the CD2 chimeras are plotted in B. All data in this figure are derived from the same transfected cell populations but are representative of at least three independent transfection experiments.

sequestration-mediated inactivation of MAP4 involved a pMEP-vector based cotransfection system that allows stringently regulated coexpression of two gene products from the hMTIIa promoter (Melander Gradin *et al.*, 1997). Immunoblot analysis of cells coexpressing MAP4 with Op18 truncation derivatives or CD2 chimeras illustrates the robustness of this genetic system (Figure 6A). Thus, the data show that coexpressed Op18-truncation derivatives or the CD2 chimeras do not influence expression levels of ectopic MAP4 and vice versa. Moreover, probing of immunoblots with anti-SLEEIQ, which recognizes a conserved region of Op18-like proteins,

Figure 5. MT destabilization in cells expressing CD2 chimeras. MT content was determined as described in MATERIALS AND METHODS, in K562 cells harboring pMEP-vector (Vector-Co) or the indicated pMEP-CD2 chimera after 20 h of Cd^{2+} -induced expression. The data are representative of three independent transfection experiments.

showed that all the ectopic truncated/chimeric derivatives are expressed at higher levels than endogenous Op18. Arbitrary quantification of ectopic proteins detected by anti-SLEEIQ relative to endogenous Op18 from serial dilutions of cell lysates indicated that truncated/chimeric derivatives were expressed at an approximately three- to eightfold higher level than endogenous Op18 (our unpublished data). However, differential efficiency of protein transfer to filters introduces a bias in the quantification of structurally different proteins by immunoblotting, which prompted us to analyze coexpressing cells by flow cytometry also (Figure 6B). The results showed that the expression levels of the Op18 truncation/CD2 chimera, as revealed by anti-SLEEIQ, are comparable in all cases. Assuming that all ectopic derivatives are detected with equal efficiency, the data presented herein indicate that each of the truncation/chimera derivatives is expressed at a 4–6-fold higher level than the level of endogenous Op18. Consistent with our previous study (Holmfeldt *et al.*, 2002), the data in Figure 6B also show that 20 h of induced expression of ectopic MT regulators does not alter the total tubulin content of K562 leukemia cells.

Analysis of net MT-polymer levels in cells coexpressing cytosolic truncated Op18 derivatives and MAP4 faithfully reproduces the results of a previous study (Holmfeldt *et al.*, 2002) in which coexpressed MAP4 counteracts MT destabilization caused by the catastrophe-promotion specific Op18(1-99)-tetraA, but not the tubulin sequestration-active Op18(25-149)-triA derivative (Figure 6C). Moreover, the results also reveal clear differences in how coexpressed CD2 chimeras modulate the MT-stabilizing activity of MAP4. Thus, ectopic MAP4 counteracts destabilization by CD2-Op18-tetraA but not by CD2-SCG10 and CD2-RB3. This is consistent with the finding that the cytosolic free tubulin concentrations are reduced by CD2-SCG10 and CD2-RB3, but not by CD2-Op18-tetraA. It follows that the results shown in Figure 6 provide independent evidence that the CD2-Op18-tetraA chimera destabilizes MTs by a sequestration-independent catastrophe-promoting mechanism similar to the previously characterized catastrophe-promotion specific Op18(1-99)-tetraA derivative.

Figure 6. Phenotypic analysis of cells coexpressing MAP4 with cytosolic or plasma membrane-targeted Op18/stathmin family members. (A) K562 cell lines were cotransfected with the indicated pMEP construct of Op18-like derivatives and either empty pMEP vector (–) or pMEP-MAP4 (+). Coexpression was induced with Cd^{2+} for 20 h and cellular lysates (20 $\mu\text{g}/\text{lane}$) were separated by 12% SDS-PAGE. An immunoblot with the indicated antibodies for detection is shown, and the arrow indicates the location of endogenous Op18 as recognized by anti-SLEEIQ. (B) The cotransfected K562 cells described in A were fixed, stained, and coexpression was quantitated by flow cytometric analysis by using the antibodies indicated. Anti-SLEEIQ antibodies allow comparison of ectopic levels of Op18-like proteins with the level of endogenous Op18. (C) MT-polymer mass of cotransfected cells was determined by flow cytometry. To exclude distortion of data due to various degrees of mitotic block, resulting from coexpression of MT-regulators, selective gating based on DNA content was used to determine MT-polymer mass specifically in G1 and S phase cells. The data are representative of three independent transfection experiments.

Spindle Formation in Cells Expressing Plasma Membrane-localized Op18/Stathmin Family Members

In contrast to the MT system during interphase, MTs of the mitotic spindle are not in physical contact with the plasma membrane (see confocal sections in Figure 8). It follows that if MT destabilization by the plasma membrane-targeted CD2-Op18-tetraA chimera requires direct physical contact, it can be predicted that this chimera will not interfere with spindle formation. Thus, a comparison of the interphase and mitotic MT-destabilizing phenotypes of CD2-Op18-tetraA would provide information on whether this MT regulator acts at a distance or requires physical MT contacts. It should be noted, however, that this comparison requires that all potential mitotic phosphorylation sites of the expressed derivatives are either substituted with Ala residues, which is the case for all Op18 derivatives, or are absent in the native protein, which is the case with the RB3 protein.

Analysis of the mitotic figures index after 20 h of induced expression shows that the catastrophe-promoting Op18(1-99)-tetraA derivative caused a somewhat increased frequency of prometaphase cells and a pronounced accumulation of abnormal mitotic spindles (Figure 7). These abnormal spindles appeared as two small star-like asters, which is consistent with our previous characterization of this truncation derivative (Holmfeldt *et al.*, 2001). The tubulin sequestration-active Op18(25-149)-triA and CD2-RB3 derivatives also caused an increased frequency of prometaphase-like spindles, but abnormal spindles were still rare. Thus, a reduced cytosolic free tubulin concentration apparently delays the formation of a mature metaphase spindle but, in contrast to catastrophe promotion, this does not result in severe spindle abnormalities. Most importantly, CD2-Op18-tetraA-expressing cells showed the same distribution of mitotic figures as vector-Co and CD2-Co expressing cells, which indicates that CD2-Op18-tetraA does not interfere at any level of mitotic spindle formation. Given that comparable expression levels of CD2-Op18-tetraA, Op18(1-99)-tetraA and Op18(25-149)-triA destabilize the interphase MT array with similar efficiency, the lack of interference with spindle formation by CD2-Op18-tetraA provides direct evidence that this plasma membrane-targeted chimera requires physical MT contact for destabilizing activity.

DISCUSSION

Reduction of Cytosolic Free Tubulin Concentrations by Tubulin Capturing at the Plasma Membrane Requires High-Affinity Heterodimer Interactions

As outlined in the INTRODUCTION, the *in vitro* activity profiles of truncated Op18 derivatives originally suggested that Op18 has the potential to destabilize MTs by both a sequestration-dependent and a specific catastrophe-promoting mechanism. Our present strategy for direct determination of reduced cytosolic free tubulin concentrations mediated by ectopic Op18/stathmin family members uses fusion proteins that target and thus localize the protein to the plasma membrane. Immunofluorescence analysis of fixed cells showed that all CD2 chimeras were detected exclusively at the plasma membrane after 20 h of induced expression from the hMTIIa promoter (Figure 3). This allowed an

Figure 7. Interference with spindle formation by “nonphosphorylatable” cytosolic or plasma membrane-targeted Op18/stathmin family members. The transfected cells described in Figure 6 were categorized with respect to frequencies of specific mitotic stages and whether the spindle appeared abnormal or not. The mitotic index of each transfected cell culture is stated within brackets to the right of each panel. Inspection was performed on cells double-stained for DNA and MTs, and the distributions of different types of mitotic cells represent the means of duplicate determinations using independent cell preparations from one transfection experiment ($n = 300$ mitotic cells). A majority of the abnormal spindles observed among Op18(1-99)-tetraA-expressing cells lacked most kinetochore MTs, and MTs looked like two small star-like asters. Most condensed chromosomes seemed aggregated in these abnormal mitotic figures (Holmfeldt *et al.*, 2001). All data in this figure are derived from the same transfected cell populations as described in Figure 6 but are representative of at least three independent transfection experiments.

Figure 8. Model of functional dichotomy of plasma membrane-targeted Op18-like proteins. (A) CD2-coexpressing K562 leukemia cells were costained with anti-CD2 (red) and anti- α -tubulin (green) and analyzed by confocal microscopy. A confocal section of a cell in interphase (left; note that the nucleus is located below the confocal section) and metaphase (right) is shown. In the spherical K562 cells, many MTs are in extensive contact with the plasma membrane during interphase but not during mitosis. (B) Depiction of how plasma membrane-targeted Op18/stathmin family members may destabilize interphase MTs either by capturing of tubulin heterodimers at the plasma membrane and consequent tubulin sequestering (I), or by direct physical contact with MT ends, which we hypothesize results in catastrophe promotion (II). It is evident that spindle-MTs are well separated from the plasma membrane during mitosis (right). This implies that CD2 chimeras can only act at a distance during mitosis, that is, by a tubulin-sequestering mechanism (I) and that a CD2 chimera working primarily via catastrophe promoting direct interactions with MTs (II) would not exhibit a mitotic phenotype.

unbiased comparison of the otherwise soluble cytosolic Op18 protein with two neural Op18-like proteins, which in their native state are primarily Golgi-associated via a hydrophobic N terminus. Immunoblot and flow cytometric analysis demonstrated that the expressed CD2-chimeras are in molar excess over cellular tubulin heterodimers. Thus, based on previous estimates of a 10 μ M cytosolic Op18 concentration in K562 cells (Larsson *et al.*, 1999), it can be deduced from the present analysis that the CD2 chimeras are expressed in the 40–60 μ M range (Figure 6B, anti-SLEEIQ). This should be compared with the estimated 23 μ M total concentration of tubulin heterodimers in K562 cells (Larsson *et al.*, 1999). Given that each CD2 chimera can be predicted to bind two tubulin heterodimers (Gigant *et al.*, 2000), these approximations suggest an approximately fourfold molar excess over total cellular tubulin heterodimers.

Herein, we have evaluated tubulin heterodimer binding by plasma membrane-targeted Op18-like proteins by using three criteria, namely, 1) retention of cellular tubulin by CD2 chimeras after cell permeabilization (Figure 2D); 2) concentrations of tubulin at the plasma membrane versus cytosol (Figures 3 and 4); and 3) functional inactivation of MAP4 (Figure 6). Given that an unknown fraction of nonpolymeric cellular tubulin heterodimers may naturally be in a sequestered state, our present estimations of reduction in free tubulin concentrations in cells expressing CD2-SCG10 or CD2-RB3 chimeras should be considered to be a minimal estimate. It was also shown that plasma membrane-targeted CD2-Op18-tetraA does not cause a detectable plasma membrane accumulation of tubulin heterodimers and consequent reduction of the cytosolic levels. Given that overexpression of both the native and N-terminally truncated Op18(25-149)-triA derivative clearly mediates tubulin sequestering, as evidenced by the lack of a counteractive MT-stabilizing activity by coexpressed MAP4 (Figure 6; (Holmfeldt *et al.*, 2002),

this may seem surprising. However, it should be noted that capturing of tubulin heterodimers at the plasma membrane requires higher affinity than formation of a soluble sequestering complex within the cytosol, because the former involves the maintenance of a concentration gradient.

Op18 has a low tubulin heterodimer-binding affinity under buffer conditions designed to mimic cytosolic conditions (pH 7.4 and 120 mM KCl; Figure 1). These results should be considered from the perspective that Op18 is highly abundant in many cell types. Thus, efficient formation of sequestering complexes by Op18 would have dramatic consequences for the MT system. For example, Op18 is expressed at a sufficient concentration in K562 cells to form ternary complexes stoichiometrically with essentially all cellular tubulin heterodimers (~ 10 μ M Op18 and ~ 23 μ M tubulin heterodimers; Larsson *et al.*, 1999). It seems that only a fraction of all tubulin heterodimers are actually in a sequestering complex with Op18 under physiological conditions, because >50% of all heterodimers are normally found within MT-polymers. In the presence of paclitaxel, which promotes polymerization, this figure increases to almost 95% of cellular tubulin in polymers (Holmfeldt *et al.*, 2002). Thus, given both the high levels of competing endogenous Op18 and insufficient tubulin heterodimer binding affinity to maintain a tubulin heterodimer concentration gradient, it is not surprising that the CD2-Op18-tetraA chimera fails to mediate detectable plasma membrane accumulation of cytosolic heterodimers.

Evidence That Op18 Destabilizes the Interphase MT Array by Direct Physical Contact

During the interphase of the cell cycle in spherical K562 cells, there is extensive contact between the plasma membrane-targeted CD2 chimeras and the MTs (Figure 8A). During mitosis, however, plasma membrane localization does

not allow interactions with the bulk of MTs, which are concentrated to the spindle. Based on these cell cycle-specific differences in the distribution of the MT system, and the observed tubulin-capturing and MT-destabilizing activities of different CD2 chimeras during interphase and mitosis, we propose the model outlined in Figure 8B. This model implies that plasma membrane-targeted Op18-like proteins destabilize MTs by two distinct mechanisms, namely, tubulin sequestering and catastrophe promotion (denoted I and II, respectively). Given that CD2-RB3 and CD2-SCG10 chimeras cause plasma membrane accumulation of a large fraction of the cellular tubulin, the observed MT destabilization during interphase (Figure 5) and increased fraction of prometaphase cells (Figure 7) can readily be explained by tubulin sequestering. Because the CD2-Op18-tetraA chimera does not capture tubulin at the plasma membrane, it follows that this plasma membrane-localized derivative will not destabilize microtubules by tubulin sequestration mechanism. However, as argued in the previous section, this does not imply that Op18 in its cytosolic form is incapable of forming soluble sequestering complexes. A key observation behind the model in Figure 8B is that the tubulin sequestration-incompetent CD2-Op18-tetraA chimera, destabilizes the interphase MT-array without interfering with spindle formation. These results suggest that the tubulin sequestration-incompetent CD2-Op18-tetraA chimera destabilizes the interphase MT array by direct interactions, which does not occur during mitosis. We hypothesize that the mechanism involves catastrophe-promotion caused by direct interactions with MTs that extend through the cytoplasm to allow direct physical interactions with the plasma membrane localized CD2-Op18-tetraA. Such a mechanism is supported by the evidence that Op18 exert specific catastrophe-promoting activity in intact cells (Howell *et al.*, 1999a; Larsson *et al.*, 1999; Holmfeldt *et al.*, 2001, 2002).

In the present study, all ectopic Op18-like proteins were expressed at comparable levels and in all cases a comparable degree of MT destabilization was observed in interphase cells (Figure 6). As expected, an excess of catastrophe-promoting activity disrupted formation of functional spindles, as evidenced by the severe spindle abnormalities caused by expression of the catastrophe-specific Op18(1-99)-tetraA derivative (Figure 7; Marklund *et al.*, 1994; Holmfeldt *et al.*, 2001). It seems remarkable, however, that expression of the CD2-RB3 chimera does not cause spindle abnormalities despite the demonstrated >35% reduction in the cytosolic free tubulin concentration and substantial destabilization of interphase MTs (Figures 4 and 5). The mild mitotic phenotype of the cytosolic tubulin sequestration-specific Op18(25-149)-triA truncation derivative also demonstrates the relative tolerance of spindle formation to lowered free tubulin concentrations (Figure 7; Holmfeldt *et al.*, 2001). The specific increase of prometaphase cells observed under tubulin-sequestering conditions indicates that establishment of a mature metaphase spindle is delayed (Figure 7). Fluorescence microscopy suggests that tubulin-sequestering conditions do not alter the MT-density of metaphase spindles (our unpublished data). Together, this implies that the primary effect of lowered free tubulin concentrations is a time delay in formation of mature metaphase spindles, but once these are formed they are fully functional. It follows that fine-

tuning of the free tubulin concentrations are not of critical importance to allow formation of functional spindles.

Functional Significance of Differential Heterodimer-binding Affinities of High-Abundance Cytosolic Op18 and Low-Abundance Compartmentalized Neural Family Members

The widespread cytosolic Op18 protein is most likely important for altering MT dynamics in response to activation of various signal transducing kinases (Melander Gradin *et al.*, 1997; Gradin *et al.*, 1998; Daub *et al.*, 2001). The neural family members are clearly also of MT regulatory importance, but their expression pattern and subcellular localization indicates that their regulatory role is exerted from confined intracellular locations during neuronal process outgrowth, sprouting, and/or regeneration (Mori and Morii, 2002). It is shown here that at physiologically relevant *in vitro* conditions, the kinetic parameters of tubulin heterodimer binding are consistent with CD2-SCG10 and CD2-RB3 having sufficient affinity to mediate accumulation of tubulin at the plasma membrane, whereas CD2-Op18-tetraA does not (Figure 1). Given that the neural RB3 and SCG10 proteins have, in their native state, a hydrophobic N terminus that is responsible for targeting intracellular membranes, primarily of the Golgi apparatus but also along dendrites and axons, including growth cones (Di Paolo *et al.*, 1997; Lutjens *et al.*, 2000; Gavet *et al.*, 2002), the present study suggest that these proteins concentrate tubulin heterodimers at targeted sites. It is notable that the cellular levels of neural-specific Op18-like proteins are low, and the level in brain cells is only ~1% of that of the abundant Op18 protein (Gavet *et al.*, 2002), which is frequently expressed at equimolar levels to tubulin heterodimers (Brattsand *et al.*, 1993; Roos *et al.*, 1993). Thus, even at complete ternary complex formation, the low-abundance neural Op18/stathmin proteins will have little impact on the cytosolic free tubulin concentration in cells.

SCG10 and RB3-mediated concentration of tubulin heterodimers to confined sites may in principle be viewed as a local sequestering phenomenon; however, a large excess of free cytosolic heterodimers will rapidly eradicate a local gradient caused by these low-abundance sequestering proteins. It is uncertain at present to what extent tubulin heterodimers in complex with SCG10 or RB3 are available for MT assembly, but it still seems conceivable that a local accumulation of tubulin is of functional significance. Based on these arguments, we propose that the neural Op18/stathmin family proteins are not likely to be important as localized sequesterers and that these proteins may have the opposite function, namely, to accumulate tubulin heterodimers at specific locations, such as Golgi, dendrites and axons, including growth cones. If this is indeed the case, these sparsely expressed neural proteins can be predicted to act by a conceptually very different general mechanism from that of the abundant soluble cytosolic Op18 protein. Thus, different heterodimer-binding properties and relative abundance and compartmentalization of Op18/stathmin family members may be key features that allow these closely related MT regulators to act by different general mechanisms in diverse cell types.

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