Global Regulation of the Interphase Microtubule System by Abundantly Expressed Op18/Stathmin

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Op18/stathmin (Op18), a conserved microtubule-depolymerizing and tubulin heterodimer-binding protein, is a major interphase regulator of tubulin monomer–polymer partitioning in diverse cell types in which Op18 is abundant. Here, we addressed the question of whether the microtubule regulatory function of Op18 includes regulation of tubulin heterodimer synthesis. We used two human cell model systems, K562 and Jurkat, combined with strategies for regulatable overexpression or depletion of Op18. Although Op18 depletion caused extensive overpolymerization and increased microtubule content in both cell types, we did not detect any alteration in polymer stability. Interestingly, however, we found that Op18 mediates positive regulation of tubulin heterodimer content in Jurkat cells, which was not observed in K562 cells. By analysis of cells treated with microtubule-poisoning drugs, we found that Jurkat cells regulate tubulin mRNA levels by a posttranscriptional mechanism similarly to normal primary cells, whereas this mechanism is nonfunctional in K562 cells. We present evidence that Op18 mediates posttranscriptional regulation of tubulin mRNA in Jurkat cells through the same basic autoregulatory mechanism as microtubule-poisoning drugs. This, combined with potent regulation of tubulin monomer–polymer partitioning, enables Op18 to exert global regulation of the microtubule system.

INTRODUCTION

Tubulin α - β heterodimers polymerize into microtubules that segregate chromosomes during mitosis and have functions related to transport, polarity, and cell organization during the interphase of the cell cycle. Microtubules switch stochastically between phases of polymerization and depolymerization, a phenomenon termed dynamic instability. This dynamic behavior is dependent on the number of nucleation sites, the concentration of tubulin heterodimers, and a diverse array of regulatory proteins (reviewed in Desai and Mitchison, 1997). One such protein, Op18/stathmin (Op18), forms ternary complexes with two tubulin heterodimers aligned head to tail (Gigant *et al.*, 2000; Steinmetz *et al.*, 2000). Op18 has been shown to destabilize microtubules by two distinct mechanisms *in vitro*, namely, by promoting catastrophes (i.e., a switch from polymerization to depolymerization) (Belmont and Mitchison, 1996) and by forming tubulin sequestering complexes (Jourdain *et al.*, 1997; reviewed in Cassimeris, 2002). The significance of these destabilizing activities *in vivo* has been suggested by the finding of reduced catastrophe promotion and increased microtubule polymerization in newt lung cells in which Op18 was inactivated by injection of inhibitory antibodies (Howell *et al.*, 1999).

Although Op18 seems to be expressed in most cells, the expression levels vary considerably between cell types and during cell differentiation, and Op18 is frequently highly

expressed in diverse types of malignancies (Hanash *et al.*, 1988; Koppel *et al.*, 1990; Brattsand *et al.*, 1993). Op18 is extensively phosphorylated on four Ser residues during mitosis (Larsson *et al.*, 1995), which serves to inhibit its destabilizing activity during spindle assembly (Marklund *et al.*, 1996; Larsson *et al.*, 1997). Op18 is also phosphorylated during the interphase in response to multiple signaling pathways (reviewed in Deacon *et al.*, 1999), which results in various degrees of functional inactivation (Horwitz *et al.*, 1997; Melander Gradin *et al.*, 1997; Gradin *et al.*, 1998). By analysis of three human leukemia/lymphoma cell types, we have found that Op18 depletion results in extensive overpolymerization of the interphase microtubule system (Holmfeldt *et al.*, 2007). Consistent with these results, gene-product replacement in the Jurkat T cell leukemia model has revealed that phosphorylation-inactivation of Op18 in response to T cell antigen receptor signaling is both necessary and sufficient for increased polymerization of tubulin (Holmfeldt *et al.*, 2007).

The basal tubulin heterodimer content is likely to be mainly regulated by the tissue-specific differential transcription of six α -tubulin genes and seven β -tubulin genes, the products of which combine to form heterodimers (reviewed in Luduena, 1998). Synthesis of stoichiometric amounts of the α - and β -tubulin subunits of the heterodimer involves translational repression of α -tubulin mRNA by excess free α -tubulin (Gonzalez-Garay and Cabral, 1996). In addition to tissue-specific transcriptional regulation, analysis of the actions of microtubule-polymerizing/depolymerizing drugs has established the existence of a posttranscriptional autoregulatory mechanism that responds to rapid alterations in the polymerization state through changes in the stability of the polysomal mRNA (Gay *et al.*, 1987; Pachter *et al.*, 1987; Theodorakis and Cleveland, 1992; Bachurski *et al.*, 1994). It is still unknown how an altered polymerization state is transduced into altered α - and β -tubulin mRNA stability, but it has been assumed that by some means, cells sense the level

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Abbreviations used: Op18, Op18/stathmin; shRNA, short hairpin RNA.

of unpolymerized tubulin (reviewed in Cleveland, 1989). However, detailed analyses of cell lines with acquired resistance to the microtubule-stabilizing drug taxol have shown that substantial changes in tubulin heterodimer content, monomer–polymer partitioning, or both may occur without compensatory changes in tubulin synthesis (Boggs and Cabral, 1987; Barlow *et al.*, 2002). Thus, the significance of autoregulation of tubulin mRNA stability for the normal regulation of tubulin heterodimer content and partitioning is still unclear, and it has been proposed that autoregulation is not activated by small changes in polymerization (Wang *et al.*, 2006).

Op18 deficiency in the developing *Drosophila* embryo has been shown recently to cause a ~2.5-fold decrease in total tubulin heterodimer content as well as reduced microtubule stability, and the authors (Fletcher and Rorth, 2007) proposed that Op18 is essential for long-term maintenance of the microtubule system. The suggestion of such an essential role of Op18 during *Drosophila* embryogenesis contrasts with the results of analysis of Op18-deficient mice, which are viable (Schubart *et al.*, 1996), and the reported phenotypes are limited to neurological defects in adult animals (Liedtke *et al.*, 2002; Shumyatsky *et al.*, 2005). Moreover, we have reported that extensive Op18 depletion of K562 cells, a human leukemia cell line, by stable expression of interfering short hairpin RNA results in a dramatic increase in interphase microtubule polymers in the absence of detectable alterations in tubulin protein content or in the density of mitotic spindles (Holmfeldt *et al.*, 2004, 2006, 2007). Nevertheless, the classical model of tubulin autoregulation outlined above suggests that a protein controlling tubulin monomer–polymer partitioning could also have the potential to control tubulin synthesis, and thus function as a global regulator of the microtubule system. Here, we address these questions by analysis of Op18-mediated control of tubulin partitioning and synthesis in two distinct human cell model systems.

MATERIALS AND METHODS

DNA Constructs

The Epstein–Barr virus (EBV)-based shuttle vectors for constitutive expression of short hairpin RNA (shRNA) targeting Op18, shRNA-Op18-443 and shRNA-Op18-OE, and the scrambled control derivative corresponding to shRNA-Op18-443, namely, shRNA-scrambled, have been described previously (Holmfeldt *et al.*, 2007). The pMEP4 shuttle vector, which directs inducible expression of FLAG epitope-tagged Op18 (Op18-F), also has been described previously (Marklund *et al.*, 1994). It was made resistant to shRNA-Op18-443-mediated suppression by introducing seven silent mutations within the 21-nucleotide (nt) targeting sequence (Holmfeldt *et al.*, 2007).

Transfections and Cell Culture

Single transfections and cotransfections of K562 and Jurkat cells using EBV-based replicating shuttle vectors and subsequent selection of hygromycin-resistant cell lines were performed as described in detail previously (Holmfeldt *et al.*, 2004, 2007). Transfection with replicating shuttle vectors that direct constitutive synthesis of specific interfering shRNA was performed according to the same basic protocol as described for pMEP vectors (Melander Gradin *et al.*, 1997), with 2 μ g of constructs producing shRNA-Op18 mixed with vector-Coup to a total quantity of 16 μ g DNA. For inducible expression of ectopic Op18 in cells synthesizing shRNA, 2 μ g of shRNA-Op18-443 was mixed with 4 μ g of pMEP-Op18-F and vector-Co DNA up to a total quantity of 16 μ g of DNA. To keep DNA concentrations constant, vector-Co DNA was used to replace shRNA-Op18-443 DNA and/or pMEP-Op18-F in control cell populations. For graded expression levels of ectopic Op18-F, cells were transfected with 0, 1, 2, 4, 8, and 16 μ g of pMEP-Op18-F mixed with the empty pMEP vectors up to a total quantity of 16 μ g of DNA. Due to the stringent replication control of the EBV-based shuttle vectors, the ratio of transfected DNAs is stable during the 5- to 7-d time course of the present experiments (Melander Gradin *et al.*, 1997). Conditional expression was induced from the human metallothionein IIA (hMTIIa) promoter of the pMEP vector by addition of Cd²⁺ as described previously (Holmfeldt *et al.*, 2007). Human peripheral

blood mononuclear cells were isolated from whole blood by density gradient centrifugation using Ficoll-Hypaque and cultured in RPMI 1640 medium containing 5% fetal calf serum in the presence of the T cell activating antibody OKT3 (0.1 μ g/ml) for 3 d. Thereafter, activated T-cells, i.e., T-blasts, were expanded in the presence of the growth factor interleukin-2 for 5–9 d before analysis.

Immunoblotting and Quantification of Immunofluorescence by Flow Cytometry

Immunoblotting and subsequent detection using the ECL detection system (GE Healthcare, Chalfont St. Giles, United Kingdom) were performed using anti-proliferating cell nuclear antigen (PCNA) (PC10, Dakopatts, Glostrup, Denmark), anti- α -tubulin (B-5-1-2; Sigma-Aldrich, St. Louis, MO), and anti-Op18 as described previously (Holmfeldt *et al.*, 2003a). Quantification of total tubulin content and Op18 by immunoblotting was performed by serial dilution of cell lysates, and concentrations were calculated according to a standard curve based on serial dilutions of purified bovine tubulin or recombinant Op18 proteins. Quantification of total tubulin content by flow cytometry was performed on cells chilled on ice, to depolymerize microtubules, followed by fixation with 4% paraformaldehyde. Fixed cells were permeabilized with saponin (0.2%), and staining was performed with anti- α -tubulin (B-5-1-2) and appropriate secondary antibodies as described previously (Holmfeldt *et al.*, 2002). Flow cytometry was performed using a FACSCalibur instrument (BD Biosciences, San Jose, CA). More than 90% of all cells were included in the acquisition gate, and >150,000 cells were collected.

Quantification of Tubulin Monomer–Polymer Partitioning

Analysis of cellular microtubule content by flow cytometry (with >95% of all cells included in the acquisition gate and >200,000 cells collected) was performed using a FACSCalibur instrument as described previously (Holmfeldt *et al.*, 2001), but with the following modifications: soluble tubulin was pre-extracted in a saponin-containing microtubule-stabilizing buffer modified by increased pH (7.0), omission of glycerol, and a reduced paclitaxel concentration (50 nM rather than 4 μ M). These modifications minimize nonspecific polymerization of microtubules during the fixation step (Holmfeldt *et al.*, 2003b). This flow cytometry-based procedure faithfully reproduced the results obtained by quantification of soluble and particulate polymeric tubulin by Western blot analysis, and it greatly increased the accuracy of determinations compared with previous methods based on Western blot analysis. To determine the proportion of dilution-resistant microtubules, soluble tubulin was pre-extracted in a buffer containing saponin as described above for standard conditions, but taxol was omitted from the extraction buffer. To determine the total amount of polymerizable tubulin, cells were treated with the polymerization-promoting drug paclitaxel (15 min; 2 μ M), which was found by quantitative Western blotting to cause essentially complete polymerization (<3% soluble tubulin), and this allowed calculation of the percentage of tubulin in polymers under the different experimental conditions.

Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted with the TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. cDNA was synthesized from 1 μ g of total RNA using the RevertAid H Minus First Strand cDNA Synthesis kit containing Moloney murine leukemia virus reverse transcriptase and random hexamer primers (MBI Fermentas, Hanover, MD). Quantitative RT-PCR of triplicate samples with a κ 1-tubulin specific primer pair (forward, 5'-ACC ATC AAA ACC AAG CGC; reverse, 5'-TGC AGG GCC AAA AGG AAT), a β 1-tubulin specific primer pair (forward, 5'-CCC CAT ACA TAC CTT GAG GCG A; reverse, 5'-GCC AAA AGG ACC TGA GCG AA), a primary β 1-tubulin transcript-specific primer pair with the 5' primer homologous to intron sequences (forward, 5'-GTG AAT CTG TCA TTT TGT CC; reverse, 5'-GCC AAA AGG ACC TGA GCG AA), or a glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific primer pair (forward, 5'-GGA AGC TTG TCA TCA ATG GAA; reverse, 5'-AGG CTG TTG TCA TAC TTC TCA) was performed using the Biotools QuantiMix EASY SYG kit and the iCycler iQ multicolor real-time PCR detection system (Bio-Rad, Hercules, CA). Cycling parameters were 95°C for 3 min, 40 amplification cycles of 95°C for 10 s and 60°C for 45 s, and a 55→95°C melting gradient (10-s inclinations of 0.5°C). The presented data were internally normalized relative to GAPDH mRNA levels. Using ubiquitin and β -actin mRNA as additional internal controls, we have confirmed that the experimental conditions do not cause variations in GAPDH mRNA levels.

RESULTS

Differential Op18 Depletion Phenotypes in Two Human Leukemia Cell Model Systems

Here, we used the K562 erythroleukemia and Jurkat T cell leukemia cell model systems to explore regulation of the

Figure 1. Quantification of Op18 and tubulin protein content in the K562 and Jurkat cell model systems. Immunoblots of total lysates of K562 cells, Jurkat cells, and normal human exponentially growing T-cells, i.e., T-blasts, by using anti- α -tubulin or anti-Op18 for detection are shown. Quantification (shown at the bottom) was achieved by serial dilution of total lysates and comparison with a standard curve of purified bovine brain tubulin or human recombinant Op18. The data plotted are representative of at least three independent analyses performed in triplicate. T-blasts from three healthy donors were analyzed, and they were found to be indistinguishable regarding Op18 and tubulin levels.

microtubule system by Op18. By immunoblot analysis, both of these cell lines have a tubulin heterodimer content corresponding to $\sim 2\%$ of the total cell proteins, which is essentially the same as for the nontransformed counterpart of the Jurkat T cell leukemia, namely, normal exponentially growing human T-blasts grown in the presence of the growth factor interleukin-2 (Figure 1). K562 and normal T-blasts have similar Op18 content, whereas Jurkat cells express approximately twofold more. Because Op18 binds two tubulin heterodimers, the present quantifications suggest that there is sufficient Op18 in Jurkat cells for complex formation with all tubulin heterodimers even under conditions of complete depolymerization, i.e., a molar ratio of Op18 to tubulin of 0.5 (see Figure 1, bottom, for calculated molar ratios). Moreover, given that $\sim 50\%$ of all tubulin heterodimers are normally polymerized into microtubules, there is also sufficient Op18 in K562 and T-blasts for complex formation with all nonpolymerized tubulin heterodimers under normal partitioning conditions.

Efficient depletion of Op18 can be achieved in Jurkat and K562 cells by means of an EBV-based replicating vector system that directs expression of Op18-specific interfering shRNA. This vector confers hygromycin resistance that, combined with high transfection efficiency, allows selection of homogeneous Op18-depleted cell lines within 3 d. Previous studies of Op18-depleted K562 cells over a 9-d period did not reveal any defect in growth rate or phenotype during spindle assembly (Holmfeldt *et al.*, 2006), and we have subsequently obtained the same results using the Jurkat cell model system (data not shown). As shown in Figure 2A, shRNA-Op18 caused efficient depletion of Op18 in both K562 and Jurkat cells ($>95\%$ specific depletion), and analysis of tubulin subunit partitioning in interphase cells showed that depletion results in increased polymerization (Figure 2B, open bars). However, the effect in Jurkat cells was consistently less dramatic than in K562 cells, in which Op18 depletion resulted in polymerization of almost all tubulin subunits into microtubules ($>96\%$ polymerization of tubulin).

Figure 2. Tubulin monomer-polymer partitioning in Op18-depleted cells. K562 and Jurkat cells were transfected as described in *Materials and Methods* with a replicating shuttle vector that directs synthesis of shRNA designed to target Op18 (shRNA-Op18), or with the empty vector (vector-Co) as indicated. Untransfected cells were counterselected with hygromycin for 5 d. (A) Immunoblots of total lysates of transfected cells obtained by using the indicated antibody for detection. The PCNA protein was used as loading control. Quantification was achieved by serial dilution, which revealed $>96\%$ specific depletion of Op18. (B) Levels of polymeric tubulin under standard assay conditions aimed at maintaining steady-state microtubule levels (open bars) and dilution-resistant polymeric tubulin (closed bars) were determined as described in *Materials and Methods*. Data represent the proportion of polymeric tubulin as a percentage of the total amount of polymerizable tubulin heterodimers, as determined by $2 \mu\text{g}/\text{ml}$ taxol treatment for 1 h. Levels of polymeric tubulin in K562 cells (C) or Jurkat cells (D) were analyzed as in panel B after 2 h in the presence of graded concentrations of the microtubule-destabilizing drug nocodazole. Closed symbols represent vector-Co transfected cells, and open symbols represent cells after 5 d of Op18 depletion. All data are the means of duplicate determinations, and they are representative of at least three independent transfection experiments.

To address the question of whether Op18 is of significance for the stability of the bulk of interphase microtubule polymers, we determined the fraction of dilution-sensitive microtubules and the sensitivity to graded doses of the microtubule-destabilizing drug nocodazole. Permeabilized K562 and Jurkat cells were found to contain similar proportions between dilution-sensitive and dilution-resistant microtubules, defined as described in *Materials and Methods*, and Op18 depletion did not have much of an effect on these proportions (Figure 2B, closed bars). Moreover, the micro-

tubules of K562 and Jurkat cells seem to be equally sensitive to nocodazole, and Op18 depletion did not significantly change the dose response of nocodazole-dependent depolymerization in either of the two cell model systems (Figure 2, C and D). Thus, Op18 depletion results in increased partitioning of tubulin into polymers without obvious changes in the general stability of the bulk of microtubule polymers.

The present system for RNA interference from a replicating vector allows analysis of phenotypes from an early time point after Op18 depletion, and over many subsequent cell cycles. Interestingly, although our results indicate that the total tubulin heterodimer content does not change in K562 cells, which is consistent with the results of previous studies (Holmfeldt *et al.*, 2006), a 25–35% decrease in total tubulin heterodimer content was observed in Jurkat cells as early as 3 d after Op18 depletion (Figure 3A). These results were confirmed using shRNAs targeting two distinct sequences of Op18 mRNA (data not shown), and we did not observe any effect from expression of a scrambled shRNA sequence. Moreover, by complementation using an epitope-tagged Op18 derivative resistant to shRNA-Op18 (Op18-Flag), we found that the observed decrease in total tubulin heterodimer content in Jurkat cells is a specific effect of Op18 depletion (Figure 3B, with the position of the ectopic Op18-Flag protein shown by an arrowhead). Hence, Op18 at endogenous levels modulates tubulin heterodimer content in Jurkat cells, which was not observed in K562 cells.

K562 and Jurkat Cells Respond Differently to Increased Op18 Content

We also performed the reciprocal experiment to Op18 depletion, namely, to explore the consequences of Op18 overexpression in K562 and Jurkat cells. We first determined the dose dependence by which ectopic Op18 depolymerizes microtubules, which was done by transfection of graded copy numbers of an EBV-based replicating expression vector as described in *Materials and Methods*. After 5 d of counterselection with hygromycin, ectopic Op18 expression was induced for 8 h from the hMTIIa promoter. As shown in Figure 4A, this genetic system allows graded increments of Op18 expression in K562 cells that covers a range between the endogenous level of $\sim 1 \mu\text{g}/\text{mg}$ total cellular proteins and approximately fivefold overexpression. In the case of Jurkat cells, which have twice as much endogenous Op18, graded increments up to an ~ 3.5 -fold overexpression level were obtained (Figure 4A). Parallel analysis of tubulin partitioning revealed that four- to fivefold overexpression of Op18 is sufficient for almost complete depolymerization in K562 cells (Figure 4A). However, the Op18 dose dependency for ectopic Op18 in Jurkat cells is quite different; whereas an ~ 1.5 -fold increase in Op18 was found to be sufficient for substantial depolymerization, it seems that about half of the microtubule content is essentially resistant to Op18-mediated depolymerization. Thus, with respect to tubulin monomer-polymer partitioning, K562 cells respond more dramatically than Jurkat cells to both Op18 depletion (Figure 2B) and high levels of overexpression (Figure 4A).

To determine the effect of elevated expression of Op18 on the total tubulin heterodimer content, transfected cells were induced to express maximal levels of Flag epitope-tagged Op18 (Op18-Flag), and they were analyzed over a 3-d period. As shown by immunoblotting, induced expression of Op18-Flag in Jurkat cells resulted in a somewhat transient peak of expression at 24 h, whereas ectopic expression levels in K562 cells were found to remain constant (Figure 4B, with the Op18-Flag protein shown by an arrowhead). Importantly, analysis of the total tubulin heterodimer content

Figure 3. Tubulin heterodimer content in Op18-depleted K562 and Jurkat cells. (A) Cells were transfected with shRNA-Op18 or a scrambled shRNA control as in Figure 2, and Op18 depletion was analyzed by immunoblotting at the days indicated. Tubulin heterodimer content of K562 cells (open symbols) or Jurkat cells (closed symbols) was determined in parallel by flow cytometric analysis of paraformaldehyde-fixed and anti- α -tubulin-stained cells and expressed as percentage of the corresponding values for vector-Co transfected cells. (B) Jurkat cells were transfected as indicated either with pMEP vector alone, with shRNA-Op18, or with a mixture of shRNA-Op18 and pMEP-Op18-F, which direct expression of a shRNA-Op18-resistant Flag epitope-tagged Op18 derivative (Op18-Flag) as described in *Materials and Methods*. Cells were cultured under conditions that allow constitutive expression of ectopic Op18-F from the hMTIIa promoter of the pMEP vector. Top, immunoblots of total cellular lysates and anti-Op18 was used to detect both endogenous Op18 (arrow) and overexpressed Op18-F (arrowhead), and PCNA was used as control for equal loading. Bottom, tubulin heterodimer content determined as described in A. The data in A represent duplicate determinations of four independent transfection experiments. Student's *t* tests indicated a significant difference from the vector control ($p < 0.01$). The data in B are representative of two independent transfection experiments.

demonstrates that increased Op18 levels after 48 h cause an $\sim 20\%$ increase in total tubulin heterodimer content in Jurkat cells. Tubulin protein turnover is very slow (~ 50 h half-life; Caron *et al.*, 1985), which is consistent with both the relatively slow increase in tubulin heterodimer content and that it remains elevated even after ectopic Op18 levels have decreased. In conclusion, the combined evidence from both depletion (Figure 3) and ectopic expression (Figure 4B) of Op18 in Jurkat cells indicates positive regulation of tubulin protein content by Op18. However, in K562 cells we did not detect changes in the tubulin heterodimer content in response to either depletion (Figure 3A) or overexpression (Figure 4B) of Op18.

Figure 4. Regulation of tubulin monomer-polymer partitioning and tubulin heterodimer content by overexpressed Op18. Cells were transfected with the replicating shuttle vector pMEP-Op18-F as described in *Materials and Methods*. After 5 d of culture, Cd^{2+} was added for 8 h to specifically induce Op18-F from the hMTIIa promoter of the pMEP vector. (A) Total Op18 content (i.e., including both endogenous Op18 and ectopic Op18-F) was quantitated by immunoblotting as in Figure 1 in K562 cells (open symbols) and Jurkat cells (closed symbols) transfected with graded copy numbers of pMEP-Op18-F. Data are plotted as percentage of polymeric tubulin of the total amount of polymerizable tubulin heterodimers, determined in parallel cultures as in Figure 2B, against the estimated total Op18 content. (B) Inducible Op18 expression was analyzed by immunoblotting, and anti-Op18 was used to detect both endogenous Op18 (arrow) and overexpressed Op18-F (arrowhead), and PCNA was used as control for equal loading. The bottom panels show tubulin heterodimer content determined as in Figure 3A. The data are representative of at least three independent transfection experiments, and they are the means of duplicate determinations. Student's *t* tests in B indicated a significant difference from the vector control ($p < 0.05$).

Autoregulation of Tubulin mRNA Stability Seems Normal in Jurkat, but Defective in K562 Cells

Screening by quantitative RT-PCR and primers specific for various α - and β -tubulin isotypes showed that mRNAs for the α 1- and β 1-tubulin isotypes were predominant (>80%) in both Jurkat and K562 cells (data not shown). To distinguish transcriptional regulation from posttranscriptional regulation of mRNA stability, we also analyzed the primary unspliced β 1-tubulin transcript. Given that primary transcripts are rapidly spliced into mature mRNA, one can predict that an unspliced mRNA will have a much shorter half-life ($t_{1/2}$) than the mature mRNA. Accordingly, treatment of Jurkat cells with the transcriptional inhibitor actinomycin D revealed that the unspliced β 1-tubulin transcript has a short half-life, of ~6 min, both in the presence and

Table 1. Half-lives of tubulin primary transcript and mature total mRNA in the absence or presence of colchicine

mRNA species	$t_{1/2}$ (h) ^a	
	Untreated	+Colchicine
Pre- β 1-tubulin	0.1	0.1
α 1-Tubulin	7.0	2.9
β 1-Tubulin	6.0	4.9

^a Jurkat cells were cultured in the presence of 5 $\mu\text{g}/\text{ml}$ actinomycin D and in the absence or presence of 1 $\mu\text{g}/\text{ml}$ colchicine for 0, 0.1, 0.25, 0.5, 1, 2, 3, 4, 6, and 8 h. RNA levels at each time point were measured by quantitative RT-PCR, and $t_{1/2}$ was calculated assuming one-phase exponential decay. Equal amounts of total RNA were added to all quantitative RT-PCR reactions. The data are representative of two independent experiments.

absence of the microtubule-depolymerizing drug colchicine (Table 1). Moreover, we found that the mature α 1- and β 1-tubulin mRNAs have a half-life of 6–7 h, which in both cases was reduced by colchicine. Thus, Jurkat cells regulate the stability of tubulin mRNAs in accordance with the post-transcriptional autoregulatory mechanism (reviewed in Cleveland, 1989).

The mechanism behind posttranscriptional regulation of tubulin mRNA stability has been elucidated by making use of the effects of microtubule polymerizing/depolymerizing drugs (Cleveland, 1989). In accordance with the results of these studies, both normal T-blasts and Jurkat cells responded to 5 h of taxol-mediated overpolymerization with an increase in α - and β -tubulin mRNA levels, whereas colchicine-mediated depolymerization had the opposite effect, namely, a similar degree of reduction in quantity of both of these mRNAs (Figure 5, A and B). The primary unspliced β -tubulin transcript in Jurkat cells remained relatively constant in the presence of either taxol or colchicine, which demonstrates posttranscriptional regulation of steady-state mRNA levels (Figure 5, D and E). In colchicine-treated normal T-blasts, we consistently observed a slight decrease in the amount of primary β -tubulin transcript, but this slight decrease cannot explain the 60–65% decrease of tubulin mRNA. Hence, the evidence from either taxol- or colchicine-treated T-blasts indicates that posttranscriptional regulation is the primary mechanism behind alterations of tubulin mRNA levels in normal human cells. In contrast to the case in T-blasts and Jurkat leukemia cells; however, drug-mediated alterations in tubulin polymerization in K562 leukemia cells did not significantly alter the levels of α - and β -tubulin mRNAs (Figure 5, C and F). Thus, whereas Jurkat cells have retained the normal posttranscriptional regulation of tubulin mRNA stability as defined by the original criteria for the acute actions of colchicine and taxol, this mechanism seems to be nonfunctional in K562 cells.

Op18 Mediates Posttranscriptional Regulation of Tubulin mRNAs in Jurkat Cells

Op18 may function as a positive regulator of tubulin heterodimer content at three distinct levels, namely, stabilization of unpolymerized tubulin heterodimers through complex formation, posttranscriptional regulation of tubulin mRNA, and increased transcription. To investigate the relative importance of these possible mechanisms, we analyzed the mature α - and β -tubulin mRNAs and also the primary unspliced β -tubulin transcript. We found reduced amounts

Figure 5. Posttranscriptional regulation of tubulin mRNAs by taxol and colchicine. Jurkat cells (A and D), normal human T-blasts (B) and E), and K562 cells (C and F) were cultured for 5 h in the absence or presence of 3 $\mu\text{g}/\text{ml}$ taxol or 1 $\mu\text{g}/\text{ml}$ colchicine. Levels of α - and β -tubulin mRNA, and primary β -tubulin transcripts were determined by quantitative RT-PCR. Tubulin mRNA and the primary transcript were internally normalized relative to GAPDH mRNA levels and plotted as percentage of untreated cells. All data plotted are the means of triplicate determinations, and they are representative of at least four independent experiments.

of tubulin mRNAs in Op18-depleted cells and also an increase in these mRNAs in Jurkat cells that were induced to overexpress Op18, which indicates positive regulation of the steady-state levels of tubulin mRNA by Op18 in Jurkat cells (Figure 6, A and C). The magnitude of alterations in tubulin mRNA content in Op18-depleted Jurkat cells and in Jurkat cells overexpressing Op18 agrees with the alterations in tubulin heterodimer content observed, which suggests that Op18 controls tubulin heterodimer content mainly by regulation of tubulin mRNAs. It should also be noted that the transient nature of increased tubulin mRNA content in cells overexpressing Op18 (Figure 6C) is consistent with the declining levels of Op18-F protein observed after a peak at 24 h after induced expression from the hMTIIa promoter in Jurkat cells (Figure 4B).

Given that the levels of β -tubulin primary transcript seemed essentially unaltered both in Op18-depleted cells (Figure 6B) and overexpressing cells (Figure 6D), our data indicate regulation by a posttranscriptional mechanism. Hence, Op18 serves as a positive regulator of tubulin expression primarily by a posttranscriptional mechanism that seems likely to involve increased tubulin mRNA stability.

To evaluate whether alterations of tubulin mRNA levels in Op18-depleted Jurkat cells is reversible, we used a gene product replacement strategy for inducible expression of Op18-Flag to restore endogenous Op18 expression levels in Op18-depleted cells. As shown in Figure 6, E and F, 9 h of induced Op18 expression was sufficient for a return to normal β -tubulin mRNA levels in the absence of detectable effects on the cognate primary transcript. Thus, the de-

Figure 6. Op18-mediated regulation of tubulin mRNA. Jurkat cells were transfected with vector-Co or shRNA-Op18 (A and B) as in Figure 2, or pMEP-Op18-F (C and D) as in Figure 4. Levels of α - and β -tubulin mRNA, and primary β -tubulin transcripts were determined by quantitative RT-PCR at the indicated time point after transfection with shRNA-Op18 (A and B) or Cd^{2+} -induced expression of Op18-F (C and D). Tubulin mRNA and the primary transcript were normalized internally relative to GAPDH mRNA levels and plotted as percentage of corresponding values for vector-Co transfected cells. (E and F) Jurkat cells were transfected either with pMEP vector alone, shRNA-Op18, or with a mixture of shRNA-Op18 and pMEP-Op18-F, which direct inducible expression of an shRNA-Op18 resistant Flag epitope-tagged Op18 derivative (Op18-Flag), as described in *Materials and Methods*. After 5 d of hygromycin selection, Cd^{2+} was added for 9 h to specifically induce Op18-F from the hMTIIa promoter of the pMEP vector. The levels of β -tubulin mRNA and primary β -tubulin transcripts were determined by quantitative RT-PCR, internally normalized relative to GAPDH mRNA levels, and plotted as percentage of corresponding values for vector-Co transfected cells. It should be noted that normalizing the RNA levels relative to total RNA, rather than to GAPDH, did not significantly alter the result presented in this figure (data not shown). All data represent the means of at least three independent transfection experiments in which quantitative RT-PCR was performed either in triplicates (mature mRNA) or as two independent sets of triplicate determinations (primary β -tubulin transcripts).

Figure 7. Excessive Op18 levels block colchicine mediated tubulin mRNA destabilization. Jurkat cells were transfected with replicating shuttle vector-co or pMEP-Op18-F (16 μ g) as in Figure 4. Op18-F expression was induced from the hMTIIa promoter for a 12-h period of which taxol or colchicine was present as indicated during the last 5 h. (A) Immunoblot of total lysates of transfected cells before treatment with taxol or colchicine. Anti-Op18 was used to detect both endogenous Op18 (arrow) and overexpressed Op18-F (arrow-head), and PCNA was used as control for equal loading. (B) Levels of β -tubulin mRNA were determined by quantitative RT-PCR. Tubulin mRNA was normalized internally relative to GAPDH mRNA levels and plotted as percentage of corresponding values for untreated vector-co cells. Normalizing mRNA levels relative to total RNA, rather than to GAPDH, did not significantly alter the result presented in this figure (data not shown). All data are the means of triplicate determinations and are representative of at least three independent transfection experiments.

creased β -tubulin mRNA level in Op18-depleted cells is reversed by induced expression of ectopic Op18 through a posttranscriptional mechanism.

Excessive Op18 Concentrations Are Required to Inhibit Tubulin mRNA Destabilization by Colchicine-mediated Depolymerization

To explore the relationship between Op18-mediated increase of tubulin mRNA levels and the previously characterized taxol- and colchicine-responsive autoregulatory mechanism, we compared the effects of these drugs on control cells with cells that either overexpress or are depleted of Op18. For overexpression, transfected Jurkat cells were induced for 12 h to express Op18-F, which resulted in 3- to 4-fold increase in Op18 concentration (Figure 7A). During the last 5 h of induced expression, cells were treated with either taxol or colchicine. Consistent with data shown above, we found that β -tubulin mRNA in vector control cells increased in the presence of taxol and decreased in the presence of colchicine, and analysis of Op18-overexpressing cells reveals the expected increase of β -tubulin mRNA (Figure 7B). Significantly, although having no effect in the presence of taxol, it is evident that overexpression of Op18 blocks the β -tubulin mRNA-destabilizing effect of colchicine (Figure 7B), which was also observed by analysis of α -tubulin mRNA (data not shown). The simplest interpretation of these data is that excessive Op18 concentrations mask the effect of colchicine-mediated depolymerization by complex formation with tubulin subunits, which would be consistent with that Op18 has potential to regulate tubulin mRNA levels through the same basic autoregulatory mechanism as taxol and colchicine.

To address whether the endogenous Op18 concentration is sufficient to significantly inhibit the tubulin mRNA destabilizing effect of colchicine by complex formation or some other potential mechanism, Jurkat cells were depleted of Op18 for 5 d, and then they were compared with vector control under conditions of a 5-h treatment with either taxol or colchicine. As shown in Figure 8B, although Op18 depletion caused the expected $\sim$ 30% reduction in β -tubulin

Figure 8. The autoregulatory range defined by the response to taxol and colchicine in control and Op18-depleted cells. Jurkat cells were transfected with vector-co or shRNA-Op18 as described in Figure 2. After 5 d of hygromycin selection, taxol or colchicine was added for 5 h as indicated. (A) Immunoblots of total lysates of transfected cells before treatment with taxol or colchicine by using the indicated antibody for detection. (B) Levels of β -tubulin mRNA were determined by quantitative RT-PCR as described in Figure 7B. All data are the means of triplicate determinations, and they are representative of at least three independent transfection experiments.

mRNA in unperturbed cells (i.e., under steady-state conditions), the mRNA levels in the presence of either taxol or colchicine remained indistinguishable from those in control cells. This was also observed by analysis of α -tubulin mRNA (data not shown). Hence, in Op18-deficient Jurkat cells there is no detectable change in the minimal or maximal range of autoregulation by drugs.

Our finding that excessive Op18 concentrations are required to inhibit the colchicine response (compare Figure 7 with Figure 8) suggests that the endogenous Op18 concentration is not sufficient to exert a significant degree of tubulin mRNA regulation through tubulin complex formation. Because this concentration is still sufficient to regulate tubulin mRNA levels in unperturbed cells (Figure 8), it seems that Op18-mediated regulation of tubulin mRNA levels under physiologically relevant conditions cannot be simply explained by tubulin complex formation.

DISCUSSION

In this study, we used two distinct leukemia cell lines to explore various levels of Op18-mediated regulation of the interphase microtubule system. Our approach relied on hygromycin-selectable replicating vectors that direct either constitutive expression of Op18-specific interfering shRNA or regulatable expression of ectopic Op18. These vector systems provide homogenous cell lines within a few days of selection and allow depletion or inducible overexpression phenotypes to be monitored over extended periods of time. Our results show that 1) Op18 is a major regulator of tubulin partitioning in both cell systems but that K562 responds even more strongly than Jurkat to both Op18 depletion and overexpression; 2) Op18 acts as a positive and reversible regulator of tubulin expression in Jurkat cells, which was not observed in K562 cells; 3) Op18 has the potential to mediate positive control of tubulin mRNA levels in Jurkat cells through the same basic autoregulatory mechanism as microtubule-poisoning drugs; and 4) this autoregulatory mechanism is defective in K562 cells, which is consistent with the idea that Op18 does not mediate positive regulation of tubulin synthesis in this cell system. These results provide an example of a microtubule-destabilizing protein that exerts posttranscriptional regulation of tubulin expression and thus the level of tubulin heterodimers that can be incorporated into microtubule polymer. Our results imply that

Op18 has potential to exert global regulation of the microtubule system, and they suggest a physiological significance of the large variations in Op18 levels observed between tissues and various developmental stages, with particularly high protein levels in neural and embryonic tissues and in diverse diagnostic groups of tumors (reviewed in Mori and Morii, 2002).

Characterization of Op18-mediated Regulation of the Microtubule System in Two Human Cell Model Systems

Despite the apparent defect in the taxol/colchicine-responsive autoregulatory mechanism, the tubulin protein levels in K562 cells are essentially the same as in Jurkat cells and normal T-blasts (Figure 1), which suggests that the basic regulation of tubulin expression does not depend much on an operational mechanism for autoregulation of tubulin mRNAs. The Op18 level in Jurkat cells is almost twice as high as in K562 cells and T-blasts, but we still found that depletion of Op18 results in an even more dramatic overpolymerization phenotype in K562 cells (Figure 2B). It is notable that Op18 depletion in K562 cells results in almost complete tubulin polymerization (Figure 2B, <4% unpolymerized tubulin), which indicates that microtubules do indeed have the potential to polymerize even at severely reduced levels of soluble tubulin subunits. Moreover, overexpression of Op18 in K562 cells results in almost complete depolymerization, whereas a significant fraction of all microtubules in Jurkat cells are resistant to excessive Op18 levels. Hence, with respect to tubulin monomer-polymer partitioning, K562 cells are clearly more responsive to alterations in Op18 levels than Jurkat, and it seems likely that this reflects quantitative and/or qualitative differences in the composition of other microtubule regulatory proteins. However, based on dilution resistance and the dose response of the destabilizing drug nocodazole (Figure 2, C and D), the overall stability of microtubule polymers in K562 and Jurkat cells seems essentially the same, and significantly, Op18 depletion and consequent overpolymerization do not significantly alter the general stability of the bulk of the polymers. Thus, despite its major influence on tubulin monomer-polymer partitioning in the present cell model systems, Op18 does not seem to be of importance for the stability of the bulk of microtubule polymers.

In accordance with the finding that manipulation of the Op18 levels in K562 cells has no effect on the tubulin heterodimer content (Figures 3 and 4), we did not detect significant Op18-mediated alterations in tubulin mRNA levels (data not shown). Given that tubulin mRNA levels in K562 cells is unaltered by taxol or colchicine (Figure 5, C and F), these results are consistent with the idea that Op18-mediated regulation of tubulin protein content depends on an operational mechanism for microtubule-poisoning drug-responsive autoregulation. To explore this aspect further, we used taxol and colchicine to define the range of maximal and minimal tubulin mRNA alterations in control and Op18-depleted Jurkat cells. The data demonstrated that Op18 depletion over a time period that allows ample time for the microtubule system to adapt, does not change the magnitude of taxol- or colchicine-mediated regulation of tubulin mRNA relative to the control GAPDH mRNA (Figure 8), or to the total RNA content (data not shown). Given that the levels of primary β -transcripts were essentially the same in control and Op18-depleted Jurkat cells (Figure 6, A and B and E and F), the simplest interpretation is that the stability of tubulin mRNA is persistently reduced in Op18-depleted cells relative to control conditions.

Role of Unassembled and Free Tubulin Concentrations in Autoregulation of Tubulin Synthesis

Molecular studies of posttranscriptional autoregulation have focused on Chinese hamster ovary (CHO) cells under conditions of drug-induced polymerization/depolymerization, and it has been assumed that these drugs act through alterations in unpolymerized tubulin subunit concentrations (reviewed in Cleveland, 1989). Note that the relevance of this mechanism for regulated mRNA stability under steady-state conditions and for specifying cellular tubulin content is still unclear. Indeed, extensive studies of selected variants of CHO cells with acquired resistance to taxol have provided examples of cell lines in which the taxol/colchicine-responsive autoregulatory mechanism does not compensate for alterations in tubulin heterodimer content and/or partitioning (Boggs and Cabral, 1987; Barlow *et al.*, 2002; Wang *et al.*, 2006). Moreover, it has also been shown that increased transcription is the major mechanism behind increased tubulin heterodimer content during pressure-induced hypertrophy of cardiac cells, and changes in mRNA stability could not be detected (Narishige *et al.*, 1999). The present study nevertheless shows that Op18-mediated regulation of tubulin synthesis in Jurkat cells is exerted primarily by a posttranscriptional mechanism. Given the associated alterations in tubulin monomer-polymer partitioning, this finding is consistent with the idea that the autoregulatory mechanism in some way senses an altered steady state of tubulin partitioning. However, since Op18 in Jurkat cells functions to increase both the fraction of unpolymerized tubulin subunits and tubulin mRNA levels, it is clear from the present data that an increase of unpolymerized tubulin subunits per se is not a negative regulator of tubulin expression.

It was recently shown that the Op18 orthologue of *Drosophila* is essential for a specific subset of microtubule-dependent processes, namely, maintenance of cell or cyst polarity (Fletcher and Rorth, 2007), which is associated with ~2.5-fold less tubulin heterodimer content in Op18-deficient larvae. The mechanism responsible for reduced tubulin heterodimer content was addressed in both second instars and isolated ovaries from embryos, which revealed a surprising complexity and suggested multiple tissue-specific mechanisms behind reduced tubulin heterodimer content (see supplemental material in Fletcher and Rorth, 2007). Nevertheless, the results from second instars prompted these investigators to suggest that reduced tubulin heterodimer content might be accounted for by a posttranscriptional mechanism, which would be consistent with our studies on Op18-depleted Jurkat cells. However, the evidence for complexity in the *Drosophila* model system may suggest that Op18 also regulates tubulin heterodimer content by alternative mechanisms.

Because the total tubulin heterodimer content in Op18-deficient second and third instars was only ~40% of the control, it seems not too surprising that the microtubule polymer content in Op18-deficient developing *Drosophila* embryos seemed markedly reduced (Fletcher and Rorth, 2007). However, quantitative data on tubulin monomer-polymer partitioning in Op18-deficient and control animals were not presented, which can be ascribed to the difficulty in generating such data in whole-animal systems. To explain why Op18 deficiency results in a decrease in both tubulin heterodimer content and microtubule polymers, a simple mechanism was proposed implying that complex formation of Op18 with tubulin is required to reduce the free tubulin concentration within the pool of unassembled tubulin (i.e., tubulin heterodimers that are not in complex with Op18),

which would in turn be required for adequate stimulation of tubulin synthesis. From the standpoint of our study, it is notable that the implications of the effects of Op18 deficiency on tubulin monomer-polymer partitioning and consequent effects on the free tubulin concentrations were not considered in the model by Fletcher *et al.* Given that endogenous Op18 has a major influence on tubulin partitioning in both Jurkat and K562 cells (Figure 2), we find such a simple model unlikely. An increased proportion of polymerized tubulin in Op18-deficient cells implies counteraction of any increase that may occur in the free tubulin concentration, and we find no obvious reason for increased steady-state concentrations of free tubulin in the absence of Op18. The present finding that endogenous Op18, despite its effect on tubulin partitioning, does not detectably influence the stability of the bulk of microtubule polymers is consistent with this line of argumentation (Figure 2).

The relative contributions of the catastrophe-promoting and tubulin-sequestering activities of Op18 in microtubule depolymerization in intact cells are still unclear (reviewed in Cassimeris, 2002). If catastrophe promotion is the main mechanism, one would predict that Op18 depletion would reduce the pool of free tubulin, which would in turn give increased tubulin synthesis rather than the observed decrease. Conversely, if tubulin sequestering is the primary mechanism, an increase in the free tubulin concentration would be expected to be neutralized immediately by increased partitioning of tubulin subunits into polymers. In addition, given the interdependence of total tubulin content, monomer-polymer partitioning, and free tubulin concentrations, it is also difficult to envisage that tubulin synthesis would be controlled solely by alterations in the free tubulin concentration. Hence, although regulation of the free tubulin concentration by Op18 seems likely to be functionally important, at least under conditions of overexpression (Figure 7), it still remains a mystery how Op18-mediated regulation of steady-state tubulin monomer-polymer partitioning is transduced into posttranscriptional regulation of tubulin mRNA levels.

Global Regulation of the Microtubule System by Abundantly Expressed Op18

Here, we have shown that despite a steady-state 25–35% decrease in tubulin heterodimer content in Op18-depleted Jurkat cells, the microtubule polymer levels are still higher than in control cells. These results from a human cell line clearly contrast with the observed decrease in microtubule polymers in Op18-deficient *Drosophila* embryos, which can probably be explained to a large extent by the ~2.5-fold decrease in tubulin heterodimer content (Fletcher and Rorth, 2007). Thus, Op18 deficiency has more dramatic effects on tubulin heterodimer content in the *Drosophila* system than we observed in the mammalian Jurkat cell line. Consistent with such differences between *Drosophila* and mammals, Op18-deficient mice reproduce normally and the reported phenotypes are limited to neurological defects in adults (Liedtke *et al.*, 2002; Shumyatsky *et al.*, 2005). Because the 25–35% decrease in tubulin heterodimer content in Op18-depleted Jurkat cells has no detectable consequences for spindle assembly or for the general growth properties of cells (data not shown), it seems conceivable that many of the cells of Op18-deficient mice may have the corresponding reduction in tubulin heterodimers without obvious developmental consequences. It also seems conceivable that this level of decrease might be the cause of, or at least might contribute to, the neurological defects reported in adult Op18-deficient mice. Future analysis of the tubulin het-

erodimer content in various tissues in Op18-deficient mice may resolve these issues.

Because almost all animal cells tested seem to regulate tubulin synthesis in response to taxol and colchicine treatment (reviewed in Cleveland, 1989), the observed defect in K562 cells may be related to its tumor phenotype. The normal tubulin heterodimer content and the apparently normal function of microtubules in both control and Op18-depleted K562 cells shows that Op18-mediated regulation of tubulin synthesis is not essential for the long-term maintenance of the microtubule system. Even so, the demonstrated potency by which endogenous Op18 may act as an interphase regulator of tubulin monomer-polymer partitioning combined with the positive regulation of tubulin synthesis found in human Jurkat cells and *Drosophila* embryos, shows the potential of Op18 as a global regulator of the microtubule system in cell types in which Op18 is abundantly expressed.

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