Modulation of Tubulin Polypeptide Ratios by the Yeast Protein Pac10p

Pablo Alvarez, Adelle Smith, James Fleming and Frank Solomon

Department of Biology and Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

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ABSTRACT

Normal assembly and function of microtubules require maintenance of the proper levels of several proteins, including the tubulin polypeptides themselves. For example, in yeast a significant excess of β-tubulin causes rapid microtubule disassembly and subsequent cell death. Even the modest excess of β-tubulin produced by genetic alterations such as deletion of the minor α-tubulin gene *TUB3* affects cell growth and can confer microtubule phenotypes. We show here that the levels of the yeast protein Pac10p affect the relative levels of the tubulin polypeptides. Cells deleted for *PAC10* have the same phenotypes as do cells that express reduced levels of α-tubulin or Rbl2p, two proteins that bind β-tubulin. Conversely, overexpression of Pac10p enhances the ability of α-tubulin or Rbl2p to suppress the lethality associated with excess β-tubulin. However, Pac10p is itself not a β-tubulin binding protein. *Pac10* null cells show a 30% decrease in the ratio of α-tubulin to β-tubulin. The results suggest that Pac10p modulates the level of α-tubulin in the cell, and so influences microtubule morphogenesis and tubulin metabolism.

EARLY steps in the microtubule assembly pathway affect proper folding of the nascent tubulin chains and their incorporation into the heterodimer. Genetic and biochemical evidence demonstrates that folding of α- and β-tubulin is mediated by the Tcp-1p chaperone complex (Gao *et al.* 1992; Yaffe *et al.* 1992; Sternlicht *et al.* 1993). *In vitro*, other factors are essential, either to finish the folding reaction or to stabilize the tubulin chains until they are dimerized (Gao *et al.* 1993; Melki *et al.* 1996; Tian *et al.* 1996). In the budding yeast *Saccharomyces cerevisiae*, mutations in presumptive chaperone complex components affect microtubule assembly and function (Ursic and Culbertson 1991; Chen *et al.* 1994). However, these studies have not fully demonstrated how tubulin chains fold and assemble in cells.

The interactions of undimerized tubulin chains may have considerable physiological significance. In yeast, genetic configurations that produce higher than wild-type ratios of β- to α-tubulin are toxic (Burke *et al.* 1989; Katz *et al.* 1990; Weinstein and Solomon 1990). Acute overexpression of β-tubulin causes rapid, quantitative microtubule disassembly and subsequently a 10^4-fold decrease in cell viability. In contrast, overexpression of α-tubulin does not cause microtubule disassembly and is only slightly toxic (Weinstein and Solomon 1990). These different properties of α- and β-tubulin imply functional differences between the two proteins. For example, perhaps β-tubulin sequences are more important for interactions between the α-β tubulin heterodimer and factors essential for microtubule assembly than are α-tubulin sequences. Consequently, excess free β-tubulin could be an effective competitive inhibitor of assembly and thus toxic (Weinstein and Solomon 1992). The molecular targets of β-tubulin toxicity are as yet unidentified.

β-Tubulin lethality is efficiently suppressed by concomitant overexpression of α-tubulin, presumably by sequestering the excess β-tubulin in heterodimer. A screen for other genes that when overexpressed would also rescue β-tubulin lethality identified three *RBL* genes; one of them, *RBL2*, suppresses β-tubulin lethality as well as overexpressed α-tubulin (Archer *et al.* 1995). Like α-tubulin, Rbl2p binds specifically to β-tubulin. Cofactor A, a protein originally identified as part of an *in vitro* assay for β-tubulin folding (Campo *et al.* 1994; Gao *et al.* 1994; Tian *et al.* 1996), is structurally and functionally homologous to *RBL2* (Archer *et al.* 1995). Cofactor A is thought to bind to a relatively unfolded form of β-tubulin; however, *in vivo* Rbl2p can bind to β-tubulin both before and after it has been incorporated into heterodimer (Archer *et al.* 1998). Thus, the available *in vivo* evidence does not clearly define a role for Rbl2p in β-tubulin folding. Indeed, Rbl2p may participate in the formation of heterodimer or it may act as a buffer of free β-tubulin.

RBL2 is a nonessential gene. Δ*rbl2* strains do display modest microtubule phenotypes. We previously showed that some α-tubulin mutants require Rbl2p for viability. Because such genetic interactions can identify functions that are redundant with or act in conjunction with those of Rbl2p, we screened for mutations in genes other than α-tubulin that are synthetically lethal with Δ*rbl2*. Here we describe one such gene, *PAC10*. This gene was previously identified in a screen for genes that are required in the absence of *CIN8*, which encodes a microtubule

Corresponding author: Frank Solomon, Department of Biology and Center for Cancer Research, Building E17-Room 220, M.I.T., Cambridge, MA 02139. E-mail: solomon@mit.edu

motor protein (Geiser *et al.* 1997). We find that *pac10* null strains display phenotypes similar to those associated with deletion of *RBL2* (Archer *et al.* 1995) or of the minor α-tubulin gene *TUB3* (Schatz *et al.* 1986b). Unlike α-tubulin and Rbl2p, however, Pac10p does not form a complex with β-tubulin. Instead, our data suggest that *PAC10* expression levels affect the ratio of α-tubulin to β-tubulin, probably by modulating the level of α-tubulin. This effect explains the several microtubule-related phenotypes of altered Pac10p levels and suggests how Pac10p may function in early steps of microtubule morphogenesis.

MATERIALS AND METHODS

Strains and microbiological techniques: Genetic manipulations and growth media were standard methods (Sherman *et al.* 1986). The strains and plasmids used in this study are listed in Table 1.

Mutagenesis and mutant isolation: We mutagenized JAY551 (Δ*rbl2*) with ethylmethane sulfonate resulting in 40% viability. We plated 60,000 cells on media lacking uracil and replica plated to 5-fluoroorotic acid (5-FOA) to select for the ability to lose the plasmid pA21A bearing *RBL2*. After this first selection we isolated 105 candidates unable to grow on 5-FOA. The 5-FOA sensitivity of eight of those strains was rescued by transformation with plasmid pJA33 bearing *RBL2* marked with *HIS3*. The strains bearing the synthetic lethal mutation were backcrossed to a wild-type strain (FSY183) and segregants were tested for the mutant allele. To test allelism between the synthetic lethal mutations and α-tubulin, we crossed mutant strains to PAY60, a derivative of DBY2282 (provided by D. Botstein, Stanford University), in which the *TUB1* locus (linked to the *TUB3* locus) is marked with *LEU2*. If the synthetic lethal mutation is in one of the α-tubulin genes, its phenotype should segregate away from the *LEU* marker.

Immunological techniques: We followed standard procedures for immunoblots and immunofluorescence (Solomon *et al.* 1992), using anti-α-tubulin antibody #345 and anti-β-tubulin antibody #206 at a dilution of 1/3500 for the immunoblots; and antibody #206 at 1/2000 for immunofluorescence (Weinstein and Solomon 1990).

Cloning of *RKS2*/*PAC10*: We used strain PAY3 (*rks2-1*) as a host to clone *RKS2*. We transformed these cells with a *S. cerevisiae* genomic DNA library on a centromeric plasmid marked with *URA3* (provided by C. Thompson and R. Young, M.I.T.). We tested the 40,000 transformants for recovered resistance to 30 μg/ml benomyl and so identified 30 candidates. We isolated the suppressing plasmids from each, and characterized the inserts by restriction mapping and so identified a region common to all the inserts. Partial DNA sequencing of that region demonstrated that it was identical to *PAC10* [(GenBank accession no. U29137 (Geiser *et al.* 1997)]. A plasmid, pPA36, carrying a 1.4-kb *Bam*HI-*Kpn*I fragment that includes the entire *PAC10* gene was created by cutting pPA1 with *Bam*HI and *Kpn*I and religating this fragment into the backbone carrying *PAC10*.

Disruption of *PAC10* and *PAC2*: To disrupt the entire *PAC10* open reading frame (ORF), we used PCR to flank the *HIS3* gene with the 5′- and 3′-noncoding regions of *PAC10* (815 bp upstream of the initiation codon and 718 bp downstream of the termination codon). The PCR primers for the 5′-noncoding region were 5′-TCAGAAGGCAATGCTGAATC-3′ and 5′-AGATCTCCAAAGAAAAATAAAGGGCA-3′; and for the 3′-noncoding region, 5′-AGATCTATGTGCGTACAGTTTTC TGC-3′ and 5′-GCAGTGGTGATGATGATTGG-3′. The two fragments were cloned into the pGEM-vector (Promega, Madison, WI), generating the plasmid pPA10. The primers create a *Bam*HI site to permit cloning of a *Bam*HI fragment carrying the *HIS3* gene. This *PAC10*::*HIS3* fragment was cut from the plasmid and transformed into wild-type diploids (FSY185). We checked the transformants for the correct integration of the disruption fragment at the *PAC10* locus by PCR. A His^+ haploid containing the desired integration was backcrossed against wild-type cells and renamed strain PAY169 (Δ*pac10*).

A similar approach was used to disrupt the *PAC2* gene. The oligonucleotides 5′-TTCTTCTGGTGCAGTCAACG-3′ and 5′-GGATCCATCTCTGAAATTCGTTTTGC-3′) were used to generate a 1050-bp domain of the 5′ region; and GGATCCCCTTT TAGATTGTAAGCGGA-3′ and 5′-CAAAGACGGTAAACTAA AACAGCA-3′) were used to generate an 800-bp fragment of the 3′ region. A Δ*pac2* haploid was renamed as strain PAY175. The Δ*cin1* strain (JFY206) was provided by J. Fleming.

Analysis of suppression of β-tubulin lethality: We transformed JAY47 with several combinations of *TUB1*, *RBL2*, and *PAC10* plasmids. To determine the extent of suppression of β-tubulin lethality, we plated the transformants to galactose (inducing) and glucose (noninducing) media. The extent of suppression is expressed as the percentage of cells growing on galactose *vs.* glucose media. We made an overexpression version of the *PAC10* gene by amplifying the coding region with PCR and cloning the fragment into the *Sal*I, *Not*I sites of a p*GAL-URA3-CEN* vector (Liu *et al.* 1992) to create pPA23 (*pGal-PAC10*). The primers for the *PAC10* gene were RKS2/*Sal*I: 5′-GTCGACTATGGACACACTGTTCAACTCCA-3′ and RKS2/*Not*I 5′-GCGGCCGCACAGACACATTATATCTTGAG-3′ creating pPA23 (p*GAL-RKS2*). The construction pPA23 was checked for its ability to rescue the benomyl supersensitivity of Δ*pac10* (PAY169) in a galactose-dependent manner. The other plasmids used in the experiment were pDK44 (*TUB1-LYS2-CEN*) and pJA33 (*RBL2-HIS3-CEN*).

Sensitivity to β-tubulin lethality: We made the diploid strain PAY224 by crossing PAY169 (Δ*rks2*) and FSY626 (*TUB2-LEU2-GalTUB2*), then sporulated to generate strains PAY231 (Δ*rks2*, *TUB2-LEU2-GalTUB2*) and PAY232 (*TUB2-LEU2-GalTUB2*). To test the effect of β-tubulin overexpression, PAY231 and PAY232 cells were grown overnight in raffinose media at 30°. At 0 hr, galactose was added to 2%, and at different time points samples were obtained. To test for viability, we counted the cells in each sample and plated them to glucose plates.

RESULTS

Identification of genes synthetic lethal with Δ*rbl2*: Cells lacking Rbl2p have conditional microtubule phenotypes, and Δ*rbl2* is synthetically lethal with specific mutant alleles of α-tubulin (Archer *et al.* 1995). The vertebrate homolog of Rbl2p, cofactor A, may be involved in folding nascent β-tubulin chains *in vitro* (Gao *et al.* 1994). To learn more about the cellular functions in which Rbl2p participates, we screened for new mutations that make this gene essential. We mutagenized a Δ*rbl2* strain bearing a *CEN* plasmid (pA21A) expressing genomic *RBL2* and the *URA3* marker (JAY551; see materials and methods for details). This screen identified eight independent strains that require wild-type *RBL2* for growth. Each of the eight strains is benomyl supersensitive (Ben^{ss}), two are cold sensitive (at 15°) and one is thermosensitive (at 37°). Backcrossing the

TABLE 1

Plasmids and strains

	Genotype	Reference
Strain		
JAY551	*MAT*α Δ*rbl2 ura3 leu2 his3 ade2* (pA21A)	Archer (1996)
FSY183	*MAT***a** *ura3 leu2 his3 lys2*	Katz *et al.* (1990)
DBY2282	*MAT*α *ura3 leu2 his3 lys2 trp1 TUB1-LEU2-TUB1*	Schatz *et al.* (1986a)
FSY185	*MAT*α/*MAT***a** *ura3/ura3 leu2/leu2 his3/his3 lys2/lys2 ade2/ADE2*	Katz *et al.* (1990)
JAY47	*MAT*α/*MAT***a** *ura3/ura3 leu2/leu2 his3/his3 lys2/lys2 ade2/ADE2 TUB2/TUB2-LEU2-GAL TUB2*	Archer *et al.* (1995)
PAY1	*MAT*α Δ*rbl2 rks2-1 ura3 leu2 his3 ade2* (pA21A)	This article
PAY3	*MAT*α Δ*rbl2 rks2-1 ura3 leu2 his3 ade2* (pJA33)	This article
PAY60	*MAT***a** *ura3 leu2 his3 lys2 trp1 TUB1-LEU2-TUB1*	This article
PAY169	*MAT***a** *pac10:HIS3 ura3 leu2 his3*	This article
PAY175	*MAT***a** *pac2::HIS3 ura3 leu2 his3*	This article
PAY223	*MAT*α/*MAT***a** *rks2-1/pac10::HIS3 ura3/ura3 his3/his3 leu2/leu2* (pA21A)	This article
PAY231	*MAT***a** *pac10::HIS3 ura3 leu2 his3 TUB2-LEU2-TUB2*	This article
PAY232	*MAT***a** *ura3 leu2 his3 TUB2-LEU2-TUB2*	This article
JAY528	*MAT*α *rbl2::URA3 ura3 leu2 his3 lys2*	Archer (1996)
PAY170	*MAT*α *pac10:HIS3 ura3 leu2 his3*	This article
JFY209	*MAT***a** *cin1:URA3 ura3 leu2 his3*	This article
Plasmid		
pA21A	*RBL2 URA3 CEN*	Archer *et al.* (1995)
pRB539	*TUB1 LEU2 CEN*	Schatz *et al.* (1986a)
pJA33	*RBL2 HIS3 CEN*	Archer (1996)
pDK44	*TUB1 LYS2 CEN*	Kirkpatrick and Solomon (1994)
pRB624	*tub1-724 LEU2 CEN*	Schatz *et al.* (1988)
pRB628	*tub1-728 LEU2 CEN*	Schatz *et al.* (1988)
pRB638	*tub1-738 LEU2 CEN*	Schatz *et al.* (1988)
pRB659	*tub1-759 LEU2 CEN*	Schatz *et al.* (1988)
pRB614	*tub1-714 LEU2 CEN*	Schatz *et al.* (1988)
pRB627	*tub1-727 LEU2 CEN*	Schatz *et al.* (1988)
pRB630	*tub1-730 LEU2 CEN*	Schatz *et al.* (1988)
pRB646	*tub1-746 LEU2 CEN*	Schatz *et al.* (1988)
pPA1,2,3	*URA3 CEN* library plasmids containing *PAC10*	This article
pPA10	pGEM *pac10::HIS3*	This article
pPA12	pGEM *pac2::HIS3*	This article
pPA23	*GAL-PAC10 URA3 CEN*	This article
pPA36	*PAC10 URA3 CEN*	This article
pPA45	*GAL-PAC2 URA3 CEN*	This article
pPA46	*TUB3-CEN-URA3*	This article

mutagenized strains to wild-type cells (FSY183) demonstrates that these conditional phenotypes are recessive. Because Δ*rbl2* is known to be synthetically lethal with specific α-tubulin mutations, we tested to determine if the double mutants could be rescued by excess α-tubulin. In all of the mutant strains, the presence of excess α-tubulin provided by genomic *TUB1* on a low-copy plasmid (pRB539) relieves the need for *RBL2*. In seven of the eight strains, the extra copy of *TUB1* also fully suppresses the Ben^{ss} phenotype, as expected if the mutation were in either of the α-tubulin genes. However, in one of the strains—PAY1—we noticed that excess α-tubulin does not completely restore wild-type growth on benomyl, especially at higher concentrations (30–40 μg/ml) of the drug. In a direct test for allelism with α-tubulin, sporulation of the diploid resulting from crossing PAY1 with a strain bearing a *LEU2* marker integrated next to *TUB1* (PAY60) demonstrates that the benomyl supersensitivity segregates independently of the *LEU2* marker. Therefore, the new mutation is unlikely to reside in either α-tubulin gene *TUB1* or *TUB3*, which are themselves linked. We provisionally named the mutated locus *rks2-1* (*R*BL2 *K*nockout *S*ynthetic lethal).

Cloning of *RKS2*: To identify the wild-type *RKS2* sequence, we transformed PAY3 (*rks2-1*, Δ*rbl2*, pJA33) with an *S. cerevisiae* genomic library marked with *URA3* and tested transformants for suppression of the benomyl supersensitivity. About 0.1% of the 4×10^4 transformants were able to grow on 30 μg/ml benomyl. Character-

ization of several of the suppressing plasmids demonstrated they contained three genomic fragments that shared a single domain. The overlapping region corresponds to a 600-bp ORF that predicts a 199aa protein of 23.1 kD. A mutation in this same sequence previously arose from a screen for genes synthetically lethal with deletion of the nonessential mitotic motor *CIN8* (Geiser *et al.* 1997). That report named the sequence *PAC10* and described two mutant alleles. Therefore, we renamed the *rks2* mutation from our screen *pac10-3*.

Phenotypes of *pac10* mutant cells: To characterize this presumptive *pac10* mutation further, and to establish that the synthetic lethal mutation is indeed allelic to *PAC10*, we removed the entire *PAC10* ORF by integrative transformation in the wild-type diploid strain FSY185. We used PCR to confirm the presence of one wild-type and one disrupted copy of *PAC10* in the resulting diploid (see materials and methods). Sporulation of these heterozygotes produced tetrads containing primarily four viable spores, and the marker identifying the *pac10* disruption segregated 2:2. Thus Δ*pac10*, like *pac10-3*, is viable. We created a diploid strain, PAY-223, designed to be heterozygous at the *PAC10* locus (Δ*pac10*/*pac10-3*), homozygous for Δ*rbl2*, and carrying wild-type *RBL2* on a low-copy plasmid marked with the *URA3* gene. Sporulation of this strain demonstrated that all segregants require the *RBL2* plasmid for viability. These results provide further evidence that *pac10-3* is indeed a mutant allele of *PAC10*. They also show that the null allele of *pac10*, like *pac10-3*, is synthetically lethal with Δ*rbl2*.

Analysis of Δ*pac10* cells demonstrates that they display the conditional phenotypes (Ben^{ss}, moderate Cs^{-} at 15°) of *pac10-3*. These phenotypes are similar to those displayed by cells containing a moderate excess of β-tubulin due to deletion of the minor α-tubulin gene, *TUB3* (Schatz *et al.* 1986b). Neither Δ*pac10* nor *pac10-3* has abnormal microtubules at either 30° or at 15°, as assessed by immunofluorescence.

Suppression of the Δ*pac10*, Δ*rbl2* synthetic lethality by mutant α-tubulins: The phenotypes associated with the *pac10* mutants—synthetic lethality with Δ*rbl2* and benomyl supersensitivity at modest (20 μg/ml) concentrations of benomyl—are largely suppressed by a low-copy plasmid bearing the major α-tubulin gene, *TUB1* (Figures 1 and 2). However, analysis of several cold-sensitive mutant α-tubulins shows that they vary in their ability to rescue these phenotypes. We transformed PAY189 cells (Δ*rbl2*, Δ*pac10*, p*CEN-RBL2-URA3*) with plasmids bearing *tub1* mutant alleles (Schatz *et al.* 1988). We assayed for the ability of these mutant genes to support growth in the absence of plasmid-borne wild-type *RBL2*. The *tub1* mutants we tested included representatives from each of the three classes originally described: those arresting with no microtubules (class 1), with too many microtubules (class 2), or with disorganized microtubules (class 3). We found that all of the

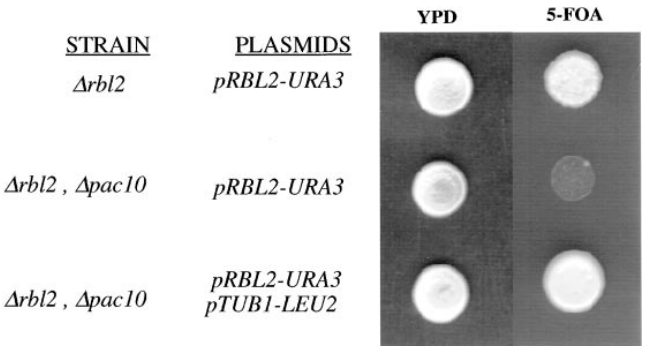

Figure 1.—The Δ*rbl2*, Δ*pac10* synthetic lethal interaction is rescued by overexpression of α-tubulin. Haploid cells bearing Δ*rbl2* and a *CEN* plasmid encoding *RBL2* and marked with the *URA3* gene (top row) can grow normally on YPD or on medium containing 5-FOA. Deletion in the same strain of the *PAC10* gene (middle row) causes these same cells to die on 5-FOA. This lethality is efficiently suppressed by the presence of a second plasmid encoding α-tubulin (third row).

mutant α-tubulins assayed can suppress the lethal phenotype at 30°, which is their permissive temperature. However, at their restrictive temperature (15°), a subset of the mutant α-tubulins do not support growth without wild-type *RBL2* (Table 2). Interestingly, the particular mutants that fail to suppress (*tub1-724*, *-728*, *-738*, and *-759*) have two other properties in common. First, all are of class I and arrest with no microtubules. Second, each of these specific α-tubulin mutations is synthetically lethal with Δ*rbl2* (Archer *et al.* 1995).

Overexpression of *PAC10* does not have an *RBL* phenotype: The requirement for either Pac10p or Rbl2p for vegetative growth could be explained if these two proteins independently carried out similar functions. To address this question, we tested if overexpressed Pac10p, like excess Rbl2p, could rescue cells from the lethality associated with excess β-tubulin. JAY47 diploid cells carry a third copy of the β-tubulin gene under the control of the inducible *GAL* promoter and integrated

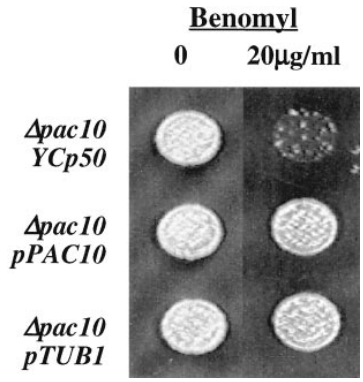

Figure 2.—The benomyl supersensitivity of Δ*pac10* cells is suppressed by overexpression of α-tubulin. Δ*pac10* cells (top row) fail to grow on solid medium containing 20 μg/ml benomyl. The presence of either *PAC10* (middle row) or *TUB1* (bottom row) on low-copy plasmids restores wild-type growth.

TABLE 2

Allele-specific suppression of the Δ*rbl2*, Δ*pac10* synthetic lethal interaction by overexpression of α-tubulin mutants at restrictive temperature

α-Tubulin gene	Suppression of Δ*pac10*, Δ*rbl2*	Viable with Δ*rbl2*[a]
TUB1	Yes	Yes
tub1-724 (1)	No	No
tub1-728 (1)	No	No
tub1-738 (1)	No	No
tub1-759 (1)	No	No
tub1-714 (2)	Yes	Yes
tub1-727 (3)	Yes	Yes
tub1-730 (2)	Yes	Yes
tub1-746 (3)	Yes	Yes

Double mutant cells (Δ*rbl2*, Δ*pac10*) were transformed with a series of *LEU2* plasmids bearing wild-type and mutated versions of the α-tubulin gene. The α-tubulin genes are listed by allele number, followed by their original classification in parentheses (Schatz *et al.* 1988; see text). The ability of each allele to suppress the synthetic lethal interaction was tested by growing at 15° on 5-FOA plates (see Figure 1). The interaction of those mutants with Δ*rbl2* previously described by Archer *et al.* (1995) is shown in the third column.

[a] Archer *et al.* (1995).

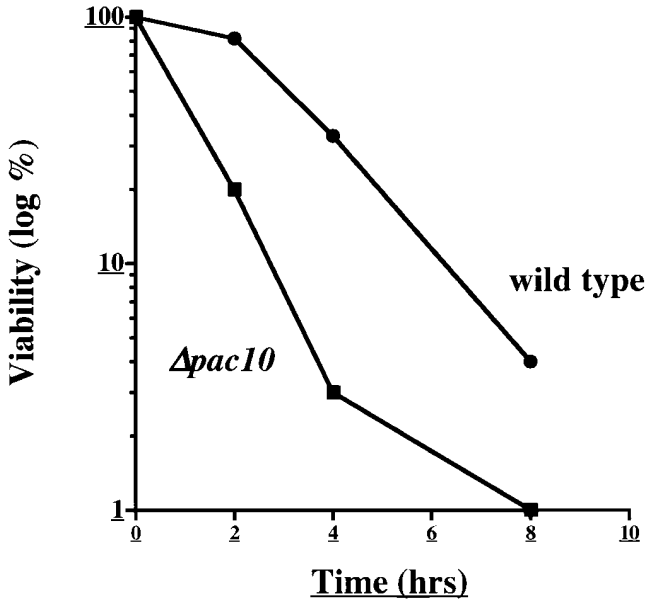

Figure 3.—Δ*pac10* cells are more supersensitive to overexpression of β-tubulin. Haploid cells, either "wild type" (PAY232) or "Δ*pac10*" (PAY231) and containing an extra copy of the β-tubulin gene under the inducible *GAL* promoter were grown overnight in selective raffinose media at 30°. At time 0 hr, galactose (final concentration, 2%) was added. At various times, aliquots of both cultures were counted for cell number, and appropriate fractions plated on glucose-containing medium. "Viability" represents the fraction of cells counted that gave rise to colonies.

at the normal *TUB2* locus. These cells die rapidly in medium containing galactose (Archer *et al.* 1995). High levels of either α-tubulin or Rbl2p rescue these cells nearly completely, and even a single extra copy of either gene provides significant rescue (10^2-fold relative to unsuppressed strains). However, *PAC10* under control of its own promoter or of the galactose promoter has no detectable effect on β-tubulin lethality.

The ability of Rbl2p to bind β-tubulin is likely to reflect some aspect of its function *in vivo.* However, we are unable to detect any physical interaction between Pac10p and β-tubulin or α-tubulin, even when both proteins are overexpressed. We searched for such complexes in extracts from cells expressing either the His_6- or HA-tagged versions of Pac10p. We analyzed those extracts using Ni-NTA beads to bind the His_6-Pac10p, or by immunoprecipitation with antibodies against the HA epitope, α-tubulin or β-tubulin. In each case, we failed to find specific association between either tubulin polypeptide and Pac10p. Both of the modified versions of Pac10p complement the *pac10* null phenotype, and therefore are functional. Under similar conditions, we can isolate Rbl2p-β-tubulin complexes (Archer *et al.* 1998) as well as the α-/β-tubulin heterodimer. These results suggest that Pac10p does not form a stable complex with β-tubulin.

Levels of Pac10p and sensitivity to β-tubulin: Several genetic and physiological experiments show that cells are sensitive to perturbations in the balance between α- and β-tubulins. Parallel analyses suggest that Pac10p levels affect that balance. First, overexpression of β-tubulin kills Δ*pac10* cells much more rapidly than wild-type cells (Figure 3). Four hours after induction of β-tubulin overexpression, viability of Δ*pac10* cells is 10-fold lower than that of wild-type cells. This supersensitivity to excess β-tubulin is comparable to that conferred by deletion of *RBL2* (Archer *et al.* 1995).

Second, increased levels of Pac10p enhance the ability of both α-tubulin and Rbl2p to rescue cells from β-tubulin overexpression (Figure 4). Typically, cells containing *GAL-TUB2* form colonies on galactose with 0.01% of the efficiency of cells plated on glucose. The presence of an extra copy of either *TUB1* or *RBL2* under control of their own promoters increases that ratio to about 2%, whereas overexpression of *PAC10* itself has no effect on survival on galactose (Figure 4). However, concomitant overexpression of *PAC10* enhances the ability of an extra copy of either *RBL2* or *TUB1* to promote growth in the presence of excess β-tubulin. When *PAC10* is present on a low-copy plasmid and under control of its own promoter, the percentage of viable colonies on galactose increases by about twofold when co-overexpressed with *TUB1* or *RBL2*. Co-overexpression of even higher levels of Pac10p, achieved using the galactose-inducible promoter, increases the viability by about eightfold. These results suggest that, although

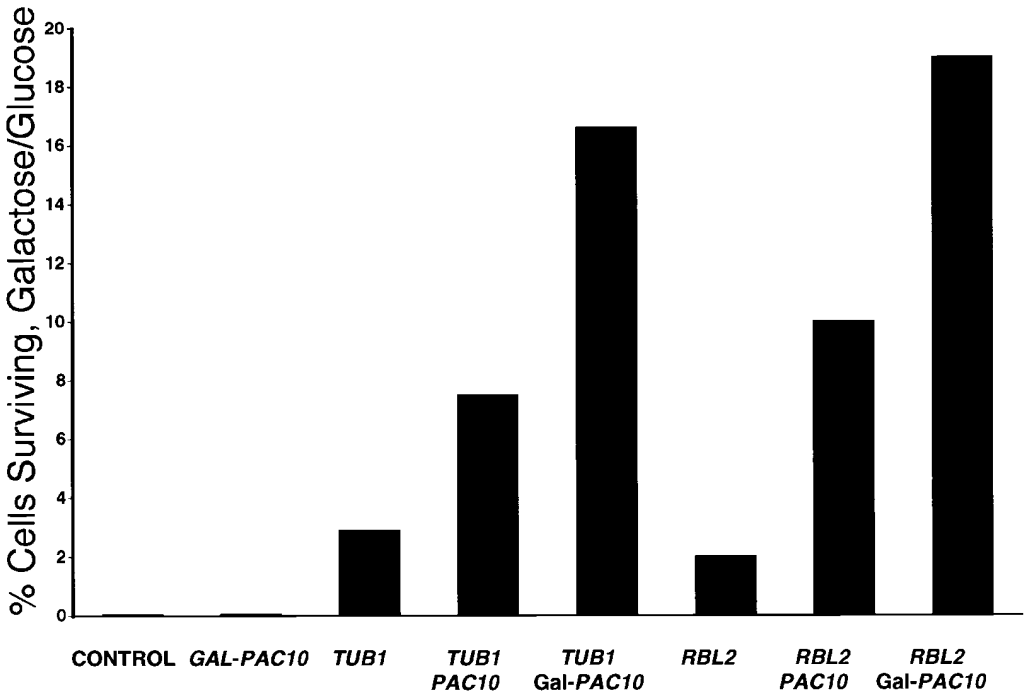

Figure 4.—Pac10p enhances suppression of β-tubulin lethality by Rbl2p and Tub1p. JAY47 cells (diploid cells containing a third intergrated copy of *TUB2* under control of the *GAL* promoter) were transformed with several combinations of the plasmids encoding *TUB1* (pRB539), *RBL2* (pA21A), *PAC10* (pPA36), and *GAL-PAC10* (pPA23) (Table 1). Aliquots were withdrawn from exponential phase cultures in glucose media, and the extent of suppression was calculated as a percentage of cells on galactose (inducing) *vs.* glucose (noninducing) plates that could form colonies. CONTROL is JAY47 transformed with YCpGAL. The control, and JAY47 cells expressing *GAL-PAC10*, gave ~0.01% colonies on galactose *vs.* glucose.

Pac10p cannot itself suppress β-tubulin lethality (Figure 4), it can enhance the ability of other genes to do so.

***PAC10* influence on tubulin levels:** Many of the consequences of altered Pac10p levels described above are consistent with the idea that Pac10p affects the activity of the tubulin chains *in vivo*. Accordingly, we measured α- and β-tubulin in Δ*pac10* and wild-type cells using immunoblots. The results of a typical experiment are shown in Table 3. We find that the levels of α- and β-tubulin are reduced in Δ*pac10* cells compared to wild type. However, the decrease in α-tubulin is greater, so the resulting ratio of α-tubulin to β-tubulin is ~30% lower in the mutants. The ratio is restored to its wild-type value when the mutant is transformed with a low-copy plasmid carrying the *PAC10* gene. This relationship between the ratio of tubulin chains and the presence of *PAC10* was found in four independent experiments.

Genetic interactions of *PAC10*: The data described in Table 3 provide a rationale for the synthetic lethality between Δ*pac10* and Δ*rbl2*. The absence of the β-tubulin binding activity of Rbl2p would be expected to enhance the cells' sensitivity to the imbalance in α-tubulin to β-tubulin produced by the absence of Pac10p. These relationships also rationalize the suppression of the Δ*pac10*, Δ*rbl2* synthetic lethality by *TUB1* (Figure 1), because an extra copy of that gene should provide more α-tubulin.

A direct test of this model is to analyze the effects of Δ*pac10* in other genetic backgrounds expected to alter the ratio of α- to β-tubulin. We showed previously that Δ*tub3* strains are viable, but supersensitive to benomyl (Katz *et al.* 1990). Tub3p contributes ~15% of the cells' α-tubulin (Schatz *et al.* 1986b), so the properties of the Δ*tub3* strain are explicable in terms of excess β-tubulin. We transformed the Δ*tub3* strain PAY290 with the *PAC10::HIS3* fragment of pPA10 and selected for strains that had stably integrated the *HIS3* marker. The majority of those isolates were unable to lose the pPA46 plasmid (p*TUB3-CEN-URA3*). We confirmed that in those strains the chromosomal copy of *PAC10* had been disrupted using PCR Southerns. In the His^+ isolates that could lose the plasmid, the disruption fragment integrated elsewhere in the genome. These results demonstrate that the double mutant Δ*pac10*, Δ*tub3* is not viable, probably because of the presence of excess β-tubulin in these cells.

TABLE 3

Absence of Pac10p decreases the α-tubulin/β-tubulin ratio in the cell

Strain	α-Tubulin	β-Tubulin	Ratio, α/β
PAC10	3.7	3.8	0.97
Δpac10, YCp50	2.1	3.3	0.63
Δpac10, pPAC10	3.1	3.2	0.93

Cultures of wild-type cells and Δ*pac10* haploids containing the indicated plasmids were grown on glucose. Protein extracts, normalized to cell number, were analyzed for α-tubulin and β-tubulin levels by immunoblotting (see materials and methods). Values were normalized to a nontubulin band in the α-tubulin immunoblot.

We also have determined whether other genes thought to participate in tubulin polypeptide metabolism interact with *PAC10*. In particular, vertebrate homologs of Cin1p and Pac2p are essential for the chaperone-mediated incorporation of denatured β-tubulin into α-/β-tubulin heterodimers *in vitro* (Tian *et al.* 1996, 1997). Disruptions of both of these genes are lethal in strains lacking the Cin8p mitotic motor (Geiser *et al.* 1997), as is Δ*pac10*. Cin1p also has been implicated in β-tubulin folding *in vivo* (Hoyt *et al.* 1997). We created diploid strains by crossing Δ*pac10* haploids (PAY170) with strains bearing deletions of either *cin1* (JFY209) or *pac2* (PAY175). The resulting diploids also contained pPA36 (p*PAC10-CEN-URA3*). Spores containing the double deletions are viable as long as the plasmid is maintained, but they are unable to grow on medium containing 5-FOA. This requirement for the *PAC10* plasmid demonstrates that both Δ*cin1* and Δ*pac2* are synthetically lethal with Δ*pac10*. A low-copy plasmid containing the *TUB1* gene rescues Δ*pac10*, Δ*pac2* and Δ*pac10*, Δ*cin1* cells (data not shown), suggesting that these synthetic lethal interactions depend at least in part on the consequences of excess β-tubulin. The results suggest that these gene products all impinge upon the same essential function, and that their proper stoichiometry is important for cell growth.

DISCUSSION

PAC10 originally was identified as a gene required for viability in the absence of the mitotic motor protein Cin8p (Geiser *et al.* 1997). However, like some of the other genes so identified, Pac10p does not appear to have motor functions, so its absence could act indirectly to exacerbate the sublethal consequences of a *CIN8* deletion. Our independent identification of *PAC10* as important for cellular functions involving the tubulin chains supports this view. The results presented above also give some insight into those functions and how *PAC10* may participate in them. Specifically, we find that cellular α-tubulin levels, and consequently the α-/β-tubulin ratio, are affected by levels of Pac10p. Previous studies demonstrate that a depressed ratio of α-/β-tubulin affects microtubule function adversely. We hypothesize that this defect in combination with either the absence of the Cin8p motor protein or of the Rbl2p β-tubulin binding protein may severely disrupt essential microtubule functions.

Levels of Pac10p are important for *in vivo* microtubule functions: In the absence of Pac10p, cells become supersensitive to the microtubule depolymerizing drug benomyl, also a property of cells that have a small deficit in α-tubulin (Katz *et al.* 1990). Similarly, *pac10* nulls are dependent upon the presence of the β-tubulin-binding protein Rbl2p for growth. Both of those phenotypes are substantially suppressed by excess α-tubulin. Those data are explicable if a consequence of the absence of Pac10p is a decrease in α-tubulin levels, and thus a reduced capacity to bind β-tubulin and so suppress its toxic effects. That deletion of *PAC10*, like deletion of *RBL2*, renders cells supersensitive to β-tubulin overexpression supports that interpretation.

However, Pac10p does not form a stable complex with β-tubulin. We cannot detect a physical association between Pac10p and either tubulin chain under conditions where we can readily isolate both Rbl2p-β-tubulin complexes and the α-β tubulin heterodimer itself. An *in vivo* test of β-tubulin binding also fails for Pac10p: even *GAL*-induced expression of *PAC10* does not increase the ability of cells to survive induced overexpression of β-tubulin under conditions in which both α-tubulin and Rbl2p act as strong suppressors. Therefore, the effect of deletion of *PAC10* on sensitivity to β-tubulin is likely exercised indirectly.

Deletion of *PAC10* changes the stoichiometry of the tubulin chains. The levels of both tubulin chains are decreased in the mutant cells, but the decline in α-tubulin is greater, so that the α/β ratio decreases by 30%. The balance of tubulin polypeptides is tightly regulated at the level of protein. For example, cells carrying a single extra copy of *TUB1* do display a proportional increase in the amount of α-tubulin mRNA, but the α-tubulin polypeptide level is very nearly the same as in wild-type cells (Katz *et al.* 1990). Presumably, the α-tubulin synthesized in these cells that is in excess of the β-tubulin complement is unstable and degraded. Perhaps in the case of Δ*pac10* cells, then, the decreased levels of α-tubulin result in undimerized β-tubulin. The diminished α/β tubulin ratio could explain the several phenotypes of *pac10* nulls and the ability of α-tubulin overexpression to suppress those phenotypes.

β-tubulin lethality and its suppression: Excess β-tubulin is much more toxic than either excess α-tubulin or excess heterodimer (Burke *et al.* 1989; Weinstein and Solomon 1990). Presumably, undimerized β-tubulin, but not undimerized α-tubulin, competes with the heterodimer for binding to factors essential for microtubule assembly and cell growth (Weinstein and Solomon 1992). This model is formally analogous to the balance of components hypothesis, which illuminated the consequences of altered stoichiometries of components in phage morphogenesis (Floor 1970; Sternberg 1976). We do not yet know the identity of the targets of free β-tubulin.

The suppression of β-tubulin lethality by α-tubulin or Rbl2p is likely to be based on their ability to bind the free β-tubulin. Although the details of these interactions are not understood, we do know that the suppression has at least one striking feature. Cells containing β-tubulin, and a low-copy plasmid bearing either *RBL2* or *TUB1* under control of their own promoters, show about 1% suppression of β-tubulin lethality, 100-fold greater than the 0.01% of the cells without the plasmid. Surprisingly, those suppressed cells form colonies the same size as those formed by wild-type cells. Clearly, then, nearly all of the products of each mitosis must be viable. If the

proportion of viable mitotic products was lower—for example, 1%—the colonies would be much smaller. This behavior may mean that once cells pass over a threshold event, they can survive excess β-tubulin. According to this model, the presence of the suppressor increases the probability that they will pass over such a threshold.

This model also provides us with a way of thinking about the effects of increased *PAC10* expression on survival of excess β-tubulin. We note that overexpression of *PAC10* enhances the suppression of β-tubulin lethality by modest increases in the levels of Rbl2p and α-tubulin, increasing survival by two- to eightfold. Although overexpression of *PAC10* is itself not sufficient to increase survival, it may provide sufficient α-tubulin to act cooperatively with excess Rbl2p or α-tubulin.

The molecular role of *PAC10*: Pac10p and Rbl2p do not appear to have redundant functions. We do not yet know in what way Pac10p acts to affect levels of α-tubulin protein. It shows no structural relationship to transcription factors, and thus is unlikely to affect α-tubulin mRNA synthesis. As noted by its original identifiers, Pac10p does have homologs in other organisms, including humans where it is believed to bind to a tumor suppressor gene product that itself has no obvious homolog in *S. cerevisiae* (Geiser *et al.* 1997). It may interact with α-tubulin mRNA or protein to stabilize them, although we have been unable to identify a stable complex with the latter. It may also be involved in folding of α-tubulin, although no homolog among the proteins essential in the *in vitro* assay for such activities is known (Tian *et al.* 1996). Experiments to distinguish among those possibilities are in progress.

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