Function of Tubulin Binding Proteins *in Vivo*

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ABSTRACT

Overexpression of the β-tubulin binding protein Rbl2p/cofactor A is lethal in yeast cells expressing a mutant α-tubulin, *tub1-724*, that produces unstable heterodimer. Here we use *RBL2* overexpression to identify mutations in other genes that affect formation or stability of heterodimer. This approach identifies four genes—*CIN1*, *CIN2*, *CIN4*, and *PAC2*—as affecting heterodimer formation *in vivo*. The vertebrate homologues of two of these gene products—Cin1p/cofactor D and Pac2p/cofactor E—can catalyze exchange of tubulin polypeptides into preexisting heterodimer *in vitro*. Previous work suggests that both Cin2p or Cin4p act in concert with Cin1p in yeast, but no role for vertebrate homologues of either has been reported in the *in vitro* reaction. Results presented here demonstrate that these proteins can promote heterodimer formation *in vivo*. *RBL2* overexpression in *cin1* and *pac2* mutant cells causes microtubule disassembly and enhanced formation of Rbl2p-β-tubulin complex, as it does in the α-tubulin mutant that produces weakened heterodimer. Significantly, excess Cin1p/cofactor D suppresses the conditional phenotypes of that mutant α-tubulin. Although none of the four genes is essential for viability under normal conditions, they become essential under conditions where the levels of dissociated tubulin polypeptides increase. Therefore, these proteins may provide a salvage pathway for dissociated tubulin heterodimers and so rescue cells from the deleterious effects of free β-tubulin.

THE spatial and temporal control of microtubule assembly is an essential aspect of many cellular functions, including division, motility, and organization of the cytoplasm. The development of a robust *in vitro* assembly reaction of microtubule polymers from heterodimeric subunits of α- and β-tubulin has had a major impact on the field. That assay led to identification of factors—structures, proteins, and small molecules—that influence the extent and organization of microtubule assembly. Reverse genetic techniques have enabled evaluation of the relevance of some of those factors to *in vivo* conditions.

Direct genetic approaches can also identify functions that directly modulate the assembly of subunits into microtubules (Pasqualone and Huffaker 1994). What is notable, however, is the growing list of gene products that affect microtubule-dependent processes but do not interact directly with the polymer. For example, several genes that affect chromosome instability (Hoyt *et al.* 1990), sensitivity to either microtubule depolymerizing drugs (Stearns *et al.* 1990) or excess β-tubulin (Archer *et al.* 1995), dependence upon a mitotic motor (Geiser *et al.* 1997), or phenotypes of mutants in γ-tubulin (Geissler *et al.* 1998) or α-tubulin (Vega *et al.* 1998) encode proteins that act on microtubule control at some step other than the polymerization reaction.

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It is also striking that the screens enumerated above have, despite their diverse designs, frequently identified the same genes. For example, certain *CIN* (*c*hromosome *in*stability) genes affect not only chromosome instability and sensitivity to benomyl—the contexts in which they originally were identified—but also yeast cells' ability to function without the kinesin Cin8p (Geiser *et al.* 1997). Similarly, mutations in the *PAC* genes perish in the absence of Cin8p, but some also participate in cellular responses to excess β-tubulin and to γ-tubulin function (Alvarez *et al.* 1998; Geissler *et al.* 1998).

Mammalian homologues of some of these proteins are essential factors in an *in vitro* reaction that mediates exchange of unfolded tubulin polypeptides into α-β-tubulin heterodimers (Gao *et al.* 1992, 1994; Melki *et al.* 1996; Tian *et al.* 1996). Under the conditions of this reaction, the tubulin polypeptides released from the chaperonin complex do not exchange efficiently into preexisting heterodimers (Tian *et al.* 1997). The assay identifies five factors that interact with monomeric α- and β-tubulin chains, finally bringing them together in a large complex that can be the precursor of heterodimer (Tian *et al.* 1997). Four of those factors have homologues in budding yeast, three identified by independent genetic investigations—Cin1p/cofactor D (Hoyt *et al.* 1990; Stearns *et al.* 1990), Rbl2p/cofactor A (Archer *et al.* 1995), and Pac2p/cofactor E (Geiser *et al.* 1997)—and a fourth, Alf1p/cofactor B identified by homology to the vertebrate protein (Tian *et al.* 1997). None of these proteins is essential in *Saccharomyces cerevisiae*, although the homologue of *CIN1* in *Schizosaccharo-*

myces pombe, ALP1, is essential and has been shown to bind microtubules (Hirata *et al.* 1998). The results suggest that the *in vitro* assay does not fully represent early steps of microtubule assembly *in vivo*.

The *RBL2* (*r*escue excess β-tubulin *l*ethality) gene has properties that make it particularly valuable for exploring the processing of tubulin polypeptides *in vivo* (Archer *et al.* 1995, 1998). Overexpression of Rbl2p efficiently rescues the microtubule disassembly and cell death that are the consequences of β-tubulin overexpression, and it interacts genetically with several conditional mutants of α-tubulin. Like its mammalian homologue cofactor A *in vitro* (Melki *et al.* 1996), Rbl2p binds β-tubulin both *in vivo* and *in vitro* to form a heterodimer that excludes α-tubulin. Unlike cofactor A, which binds only to a form of β-tubulin that is not competent to bind α-tubulin, Rbl2p can bind to β-tubulin molecules both before and after they have been incorporated into heterodimer (Archer *et al.* 1998). *RBL2* is not essential for mitotic growth but is essential for normal meiosis and normal resistance to microtubule depolymerizing drugs. In addition, its synthesis may be upregulated at the G2/M stage of the cell cycle although β-tubulin expression is apparently unchanged (Velculescu *et al.* 1997).

These properties suggest that Rbl2p functions may affect processes other than folding of β-tubulin, and the genetic interactions indicate what those processes may be. Deletion of *RBL2* is lethal in cells that have depressed ratios of α- to β-tubulin, either because they lack the minor α-tubulin gene, *TUB3* (A. Smith, M. Magendantz and F. Solomon, unpublished results) or because they lack *PAC10*, which regulates that ratio (Alvarez *et al.* 1998; Geissler *et al.* 1998). Conversely, overexpression of *RBL2* is lethal in cells expressing a mutant α-tubulin that forms a weaker α-β-tubulin heterodimer (Vega *et al.* 1998).

To understand those interactions, we have applied *RBL2* overexpression to identify nontubulin genes that influence heterodimer stability. We show here that cells lacking either of two of the proteins noted above, Cin1p/cofactor D or Pac2p/cofactor E, die upon overexpression of Rbl2p. Physiological and biochemical analyses presented here support a role for these proteins in promoting heterodimer formation. That role could be important for *de novo* heterodimer formation. However, since these genes are not essential under normal conditions, they may instead participate in a salvage pathway acting on dissociated tubulin polypeptides to protect cells from the toxicity of free β-tubulin.

MATERIALS AND METHODS

Strains, plasmids, and media: All yeast strains are derivatives of FSY185 (Weinstein and Solomon 1990) with the exception of the *tub1* mutants (Schatz *et al.* 1988). We used standard methods for yeast manipulations (Schatz *et al.* 1986; Solomon *et al.* 1992). All the relevant strains are listed in Table 1.

Screen for *erl* mutants: Wild-type cells containing *pGAL-RBL2:URA3:CEN* (pA5) were mutagenized with ethyl methanesulfonate (EMS) to 25% survival. The mutagenized strains were grown in YPD media for 4 hr. The cells were frozen at −70° in 25% glycerol. Cells were plated from frozen stocks to SC −ura glucose plates (∼200/plate) and after ∼40 hr growth were replica plated to SC −ura galactose plates. Replica plated colonies that were unable to grow on galactose were retested by streaking to SC −ura galactose and SC −ura raffinose plates. Cells unable to grow on galactose were streaked to SC 5-fluoroorotic acid (5-FOA) plates to select for loss of the plasmid. Positive *erl* (*e*nhancer of *R*bl2p *l*ethality) mutants were able to grow on galactose after loss of the pA5 plasmid, but were unable to grow on galactose after retransformation with the same pA5 plasmid.

Construction of *CIN1*, *PAC2*, and *TUB1* knockouts: To disrupt *CIN1*, the primers 5′-GCACGACGTCGATAATATTTTT GGAAAGAACGCC and 5′-GCAGAGATCTGTTGATCGCGG CAATCGTCTGTTGGTGC were used to amplify DNA in the 5′ untranslated region (UTR) of *CIN1*. The primers 5′-GAC CGTCGACGAGATAAAGAAATGCGGAATGAAGC and 5′-GACCGCATGCGAGATAAAGAAATGCGGAATGAAGC were used to amplify DNA in the 3′ UTR of *CIN1*. The PCR products from these primers were ligated into pNKY51 (Alani *et al.* 1987) on opposite ends of the *hisG-ura3-hisG* sequence. The plasmid was digested with *Aat*II and *Eag*I and the *CIN1* 5′ UTR-*hisG-URA3-hisG-CIN1* 3′ UTR DNA fragment was isolated (Qaiex II from QIAGEN, Chatsworth, CA) after electrophoresis on a 1% agarose gel. This DNA was then transformed into FSY185 to create a disruption of the entire *CIN1* open reading frame (ORF). The disruption was confirmed by Southern blot analysis of the diploids and of their haploid segregants and by the phenotypic analysis of the haploid segregants.

To disrupt *PAC2*, pPA14 containing 1050 bp of 5′ *PAC2* UTR and 800 bp of 3′ *PAC2* UTR (Alvarez *et al.* 1998) joined together at a *Bam*HI site in pGEM (Promega, Madison, WI) was digested. The *Bam*HI-*Bgl*II fragment containing *hisG-ura3-hisG* from PNK51 was cloned into the *Bam*HI site of digested pPA14. The resulting plasmid pLV59 was digested with *Bgl*II and *Not*I to release a 5.7-kb disruption fragment to transform FSY183. The disruption was confirmed by PCR analysis.

To disrupt *TUB1*, the primers 5′-CGCGACGTCTATCAATG GCGGGCAC and 5′-GTTTACAGATCTTGGGTGGC were used to amplify DNA in the 5′ UTR of *TUB1*. The primers 5′-GCGGCATGCCGACTCATACGCTGAGG and 5′-GCGC GGCCGGCATCAACTGTGACATCG were used to amplify DNA in the 3′ UTR of *TUB1*. The PCR products from these primers were ligated into pNKY51 (Alani *et al.* 1987) on opposite ends of the *hisG-ura3-hisG* sequence. The plasmid was digested with *Aat*II and *Eag*I and the *TUB1* 5′ UTR-*hisG-URA3-hisG-TUB1* 3′ UTR DNA fragment was isolated (Qaiex II from QIAGEN) after electrophoresis on a 1% agarose gel. This DNA was then transformed into FSY182 to create a disruption of the entire *TUB1* open reading frame marked with *hisG-URA3-hisG*. The disruption was confirmed by loss of HIS^+ and by PCR analysis.

Viability measurements: JFY203 (Δ*cin1* containing pA5), JFY3 (wild-type containing pA5), and LTY500 (Δ*pac2* containing pA5) were grown overnight in SC −ura raffinose media. Log phase cells were then induced with 2% galactose and at various time points aliquots of cells were taken and counted using a hemocytometer. Known numbers of cells were then plated to SC −ura glucose plates. Cell viability was measured as the percentage of cells able to form colonies on the SC −ura glucose plates.

***In vivo* His_6-Rbl2p-β-tubulin association experiments:** Yeast

TABLE 1

Strains and plasmids

Strain/plasmid	Genotype	Reference
Strains		
FSY183	*MAT***a** *his3*Δ*200 leu2-3,112 lys2-801 ura3-52* (pA5)	Weinstein and Solomon (1990)
FSY182	*MAT***a** *his3*Δ*200 leu2-3,112 lys2-801 ura3-52* Δ*tub1::HIS3* Δ*tub3::TRP1* (pRB539)	Schatz *et al.* (1988)
FSY157	*MAT***a** *his3*Δ*200 leu2-3,112 lys2-801 ura3-52* Δ*tub1::HIS3* Δ*tub3::TRP1* (pRB624)	Schatz *et al.* (1988)
JFY3	FSY183 plus pA5	This study
JFY4	FSY183 plus YCpGAL	This study
JFY5	*MAT***a** *his3*Δ*200 leu2-3,112 lys2-801 ura3-52 erl1-1* (pA5)	This study
JFY209	*MAT*α *his3*Δ*200 leu2-3,112 lys2-801 ura3-52* Δ*cin1::hisG:URA3:hisG*	This study
LTY467	*MAT*α *his3*Δ*200 leu2-3,112 lys2-801 ura3-52* Δ*pac2::hisG:URA3:hisG*	This study
JFY206	*MAT*α *his3*Δ*200 leu2-3,112 lys2-801 ura3-52* Δ*cin1::hisG*	This study
JFY203	JFY206 with pA5	This study
JFY232	Δ*rbl2::hisG,* Δ*cin1::hisG, his3*Δ*200 leu2-3,112 lys2-801 ura3-52* (p18c)	This study
JFY234	Δ*rbl2::hisG,* Δ*cin1::hisG, his3*Δ*200 leu2-3,112 lys2-801 ura3-52* (pA1A5)	This study
LTY500	*MAT*α *his3*Δ*200 leu2-3,112 lys2-801 ura3-52* Δ*pac2::hisG* (pA5)	This study
LTY576	FSY 183 with pGRH	This study
JFY252	JFY206 with YCpGAL	This study
JFY253	JFY206 with pGRH	This study
LTY503	*MAT*α *his3*Δ*200 leu2-3,112 lys2-801 ura3-52* Δ*pac2::hisG* (pGRH)	This study
JFY474	*MAT***a** *his3*Δ*200 leu2-3,112 lys2-801 ura3-52* Δ*cin1::hisG* Δ*tub1::HIS3* Δ*tub3::TRP1* (pA1A5106)	This study
LTY479	*MAT***a** *his3*Δ*200 leu2-3,112 lys2-801 ura3-52* Δ*pac2::hisG* Δ*tub1::HIS3* Δ*tub3::TRP1* (pTUB3 URA3 2u)	This study
JFY268	FSY157 with pJF10	This study
JFY269	FSY157 with YCpGAL	This study
JFY470	FSY182 with pJF15	This study
JFY471	FSY182 with YCpGAL	This study
JFY475	FSY 182 with pJF10 and pJF16	This study
JFY476	FSY 182 with pJF10 and pRS317	This study
JFY477	FSY 182 with YCpGAL and pJF16	This study
LTY564	FSY182 with pLV62 and YCpGAL	This study
LTY565	FSY182 with pLV62 and pJF10	This study
LTY566	FSY182 with pLV62 and pJF14	This study
LTY567	FSY182 with pRS317 and pJF14	This study
JFY478	FSY182 with YCpGAL and pLV71	This study
JFY481	FSY182 with pJF15 and pLV71	This study
LTY498	JFY206 with pLV56	This study
LTY597	FSY183 with pLV56	This study
JFY531	*MAT***a** *his3*Δ*200 leu2-3,112 lys2-801 ura3-52* Δ*tub1::hisG* Δ*tub3::TRP1* (pRB624)	This study
Plasmids		
YCpGAL	*CEN URA3*	
YCP50	*CEN URA3*	
p18C	*CIN1 CEN URA3*	This study
pA21A	*RBL2 CEN URA3*	Archer *et al.* (1995)
pRS316	*CEN URA3*	Sikorski and Hieter (1989)
pA5	*GAL1 RBL2 CEN URA3*	Archer *et al.* (1995)
pGRH	*GAL1 RBL2-HIS*$_6$ *CEN URA3*	Archer *et al.* (1998)
pRB624	*tub1-724 CEN LEU2*	Schatz *et al.* (1986)
pRB539	*TUB1 CEN LEU2*	Schatz *et al.* (1986)
pJF10	*GAL1 CIN1 CEN URA3*	This study
pJF14	*GAL1 CIN1-HA CEN URA3*	This study

(*continued*)

TABLE 1

(Continued)

Strain/plasmid	Genotype	Reference
Plasmids		
pJF15	*GAL1 CIN1-His$_6$-HA CEN URA3*	This study
pLV62	*GAL1 PAC2-His$_6$-HA CEN LYS2*	Vega *et al.* (1998)
pLV56	*GAL1 PAC2-His$_6$-HA CEN URA3*	Vega *et al.* (1998)
pJF16	*GAL1 PAC2 CEN LYS2*	This study
pJF17	*GAL1 CIN1 CEN HIS3*	This study
pAS56	*GAL1 CIN2 CEN URA3*	This study
pJF18	*GAL1 CIN4 CEN URA3*	This study
pLV71	*GAL1 PAC2-HA CEN LYS2*	This study
pRS313	*CEN HIS3*	This study
pRS317	*CEN LYS2*	Sikorski and Hieter (1989)
pRB316	*TUB3 2μm URA3*	Sikorski and Hieter (1989)

strains LTY503(Δ*pac2*), JFY253(Δ*cin1*), and LTY573 (wild-type) containing a *GAL-RBL2-HIS$_6$* plasmid (pGHR) were grown overnight at 30° in selective media containing raffinose to about 2×10^9 cells (log phase) per experiment. To induce *His$_6$-RBL2* expression, 2% galactose was added. After 3 hr, protein was harvested by glass bead lysis in 1 ml PME buffer plus protease inhibitors. We applied 0.85 ml of protein extract to 50 μl Ni-NTA beads (QIAGEN). We washed and eluted the bound proteins as previously described (Magendantz *et al.* 1995). Eluted proteins were subjected to SDS-PAGE analysis and probed by immunoblotting for α-tubulin, β-tubulin, and Rbl2p.

Immune techniques: *Immunoblots:* Modifications of standard procedures (Solomon *et al.* 1992) were used to assay for Rbl2p-His$_6$-β-tubulin association. After gel electrophoresis and transfer to nitrocellulose membranes, we blocked with TNT (0.025 m Tris, pH 7.5, 0.17 m NaCl, 0.05% Tween-20) for 30–120 min. Primary antibodies were incubated for >12 hr at 1/3500 (#206 or #345; Weinstein and Solomon 1990) or at 1/100 (#250; Archer *et al.* 1995) and then washed five times (5 min each) in TNT. Bound antibody was detected by ^{125}I Protein A (New England Nuclear, Boston).

In other experiments, after gel electrophoresis (as above), we blocked with milk (5% Carnation) TBST (0.05 m Tris, pH 8.0, 0.15 m NaCl, 0.1% Tween-20) overnight. Primary antibodies were incubated for 1–2 hr at 1/3500 for #206 and #345 and at 1/5000 for 12CA5 (Boehringer Mannheim, Indianapolis) in milk TBST. The blots were washed six times (two 20 sec, one 15 min, three 5 min) in TBST alone. Blots were incubated with 1/3000 dilutions of horseradish peroxidase conjugated goat anti-rabbit (Jackson ImmunoResearch, West Grove, PA) for #206 and #345 and horseradish peroxidase conjugated rabbit anti-mouse (Jackson ImmunoResearch) for 12CA5, in milk TBST, washes were done in TBST as above, and detected by chemiluminescence (Renaissance NEN).

Immunofluorescence: We used standard techniques (Solomon *et al.* 1992). Primary antibody was #206 (anti-β-tubulin) and secondary antibody was fluorescein conjugated goat anti-rabbit IgG (Cappel). To visualize DNA, 4′,6-diamidino-2-phenylindole (DAPI; Boehringer Mannheim) was used.

Analysis of α-tubulin mutations synthetic lethal with Δ*cin1* and Δ*pac2*: Δ*cin1* Δ*tub1* Δ*tub3* (JFY474) or Δ*pac2* Δ *tub1* Δ*tub3* (LTY479) strains containing a plasmid with a genomic copy of *TUB1* on a *URA3 CEN* vector or *TUB3* on a *URA3 2* μm vector, respectively, were transformed with *LEU2 CEN* plasmids containing the various α-tubulin mutations. The strains containing both the wild type and a mutant form of *TUB1* were grown on 5-FOA plates to select for cells that have lost the wild-type α-tubulin plasmid, since 5-FOA kills *URA3*$^+$ but not *ura3*$^-$ cells. Δ*pac2* or Δ*cin1* strains that are synthetic lethal with the α-tubulin mutations will be unable to lose the wild-type α-tubulin plasmid and cannot survive on 5-FOA. However, strains that are viable without the wild-type α-tubulin allele are able to lose this plasmid along with the *URA3* gene and form colonies.

Construction of *GAL-CIN1*, *GAL-CIN2*, and *GAL-CIN4*: To construct the *GAL-CIN1 URA3 CEN* plasmid, the *CIN1* ORF and additional 5′ and 3′ UTR were amplified by PCR. The 5′ primer (5′-GACACGCGTCATGAACAATATTCGGGCCT TGC) contained a *Mlu*I site and the 3′ primer (5′-CAGCCGC GGATTATATGTAAAATTTGCCGTTTAC) contained a *Sac*II site. The PCR product was ligated into the pT7-Blue plasmid from Novagen. This DNA was then digested with *Mlu*I and *Sac*II and ligated into the pRS316-GAL1 plasmid (Liu *et al.* 1992). The construct (pJF10) suppressed the benomyl supersensitive phenotype of cells deleted for *CIN1*.

To construct the *GAL-CIN1 HIS3 CEN* plasmid, the pJF10 plasmid was digested with *Apa*I and *Sac*II (both cut in the polylinker), which liberates a fragment that contains the *GAL* promoter and the entire *CIN1* open reading frame, and ligated into pRS313. The construct (pJF17) suppresses the benomyl supersensitive phenotype of cells deleted for *CIN1*.

To construct the *GAL-CIN2 HIS3 CEN* plasmid, the *CIN2* ORF and additional 3′ UTR were amplified by PCR. The 5′ primer (TAGGCCGTCGACATGGACTTTACTGCGAAGAT AAAGGGTA) contained a *Sal*I site and the 3′ primer (CGAC TAGCGGCCGCCTATAAGTAAGCGCGAAACAACTGCA) contained a *Not*I site. The PCR product was digested with *Sal*I and *Not*I and ligated into pRS316-GAL1 plasmid (Liu *et al.* 1992). The contruct (pAS56) suppresses the benomyl supersensitivity of cells deleted for *CIN2*.

To construct the *GAL-CIN4 HIS3 CEN* plasmid, the *CIN4* ORF and additional 5′ and 3′ UTR were amplified by PCR. The 5′ primer (GCCGGATCCATGGGACTACTAAGTATTA TC) contained a *Bam*HI site and the 3′ primer (CGGCC GCGGGTAATGAACTATCACGC) contained a *Sac*II site. The PCR product was digested with *Bam*HI and *Sac*II and ligated into pRS316-GAL1 plasmid (Liu *et al.* 1992). The construct (pJF18) suppresses the benomyl supersensitivity of cells deleted for *CIN4*.

Characterization of Δ*cin1*, Δ*rbl2* double mutants: A Δ*cin1::hisG/CIN1*, Δ*rbl2::hisG-URA3-hisG/RBL2* diploid containing a *RBL2* covering plasmid marked by *HIS3* was sporulated. The *cin1* null allele was followed by its benomyl supersensitivity and the *rbl2* deletion allele by the *URA3* marker. Haploid segregants harboring both null alleles and the covering plasmid were transformed with either a *pCIN1 URA3* or a *pRBL2 URA3* plasmid. Cells were grown in YPD, plated to SC −ura glucose plates, and replica plated to SC −his glucose plates. Strains that had lost the *HIS3*-marked plasmid were plated to 5-FOA plates to select for loss of the *URA3*-marked covering plasmid.

Interactions of α-tubulin mutant alleles with overproduced *CIN1*: Δ*tub1*, Δ*tub3* strains containing mutant alleles of the *TUB1* gene on *LEU2:CEN* plasmids (listed in Table 2) were transformed with pJF10 and YCpGAL. These strains were grown to saturation overnight in SC −ura glucose liquid media. The cultures were serial diluted in 96-well dishes and spotted onto SC −ura galactose plates containing 10 μg/ml benomyl, onto SC −ura galactose plates incubated at 25° (a semipermissive temperature for the growth of *tub1-724* mutant strains), and also onto galactose and glucose plates at 30° as a growth control.

Effect of *CIN1* overproduction on excess Pac2p *tub1-724* lethality: Δ*tub1*, Δ*tub3* strains containing the *tub1-724* mutant α-tubulin allele on a *LEU2 CEN* plasmid were transformed with the following plasmids: *pGAL-CIN1 CEN URA3* and *pGAL-PAC2 CEN LYS2* (JFY475), *pGAL-CIN1 CEN URA3* and *pCEN LYS2* (JFY476), or *pCEN URA3* and *pGAL-PAC2 CEN LYS2* (JFY477). The strains were grown overnight in SC −lys −ura glucose liquid media. The cultures were serial diluted in 96-well dishes and spotted onto SC −lys −ura galactose plates. Cells were also spotted onto glucose plates as a growth control.

Overproduction of combinations of the *CIN* genes in *tub1-724*: JFY531 was transformed with the following combinations of plasmids: *pGAL-CIN1 CEN HIS3* and *pGAL-CIN2 CEN URA3* (JFY525), *pGAL-CIN1 CEN HIS3* and *pGAL-CIN4 CEN URA3* (JFY526), *pGAL-CIN1 CEN HIS3* and *pGAL CEN URA3* (JFY527), *pCEN HIS3* and *pGAL-CIN2 CEN URA3* (JFY528), *pCEN HIS3* and *pGAL-CIN4 CEN URA3* (JFY529), or *pCEN HIS3* and *pGAL CEN URA3* (JFY530). The strains were grown overnight in SC −his −ura −leu glucose liquid media. The cultures were serial diluted in 96-well dishes, spotted onto SC −his −ura −leu galactose plates, and incubated at various temperatures. Cells were also spotted onto glucose plates as a growth control.

Construction of *GAL-CIN1-HA* and *GAL-CIN1-HA-His$_6$*: The 3′ third of the *CIN1* open reading frame was amplified using PCR. The 5′ primer (5′-GATGTAGGACGTCTGGTAAGAA TACAGGC) contained an *Aat*II site. Two 3′ primers were used. To make the GAL-CIN1-HA construct we used the 3′ primer (5′-CTCACCGCGGCTAGCGGCCGCCTAAAGTGATATCAG ACTCTAATATATTCGC) containing a *Not*I site followed by two stop codons and a *Sac*II site. To make GAL-CIN1-HA-His$_6$ we used the 3′ primer (5′-CTCACCGCGGCTAGTGATGG TGATGGTGATGGCGGCCGCCTAAAGTGATATCAGACTC TAATATATTCGC) containing six in-frame histidine residues, a *Not*I site, two stop codons, and a *Sac*II site. The PCR products were ligated into pT7-Blue plasmid (Novagen). The *Aat*II *Sac*II fragments were then ligated into pJF10 to create pJF11 and pJF12, respectively. A 111-bp *Not*I fragment containing the triple HA epitope from B2385 (provided by G. Fink, MIT) was cloned into the *Not*I site of pJF11 and pJF12 to create pJF14 and pJF15, respectively. These alleles of *CIN1* rescue the benomyl phenotype of Δ*cin1* cells and suppress the *tub1-724* phenotypes as well as does the unmodified *CIN1*.

***In vivo* Cin1p-HA-His6 and Pac2p-HA-His6 association experiments:** We grew yeast strains overnight in selective raffinose media at 30°. Galactose (2%) was added to induce the tagged constructs for ~4 hr. A total of 6.0×10^9 cells (log phase) were harvested by glass bead lysis per experiment in 1.1 ml PME buffer plus protease inhibitors. We applied 1 ml of protein extract to 25 μl Ni-NTA beads. We washed and eluted the bound proteins as previously described (Magendantz *et al.* 1995). Eluted proteins were subjected to SDS-PAGE analysis and probed for α-tubulin, β-tubulin, and HA(12CA5). For Cin1p-β-tubulin association experiment we used strains JFY470 (*pGAL1-10 CIN1-His$_6$-HA CEN URA3*) and JFY471 (YCpGAL). For Pac2p-Cin1p association experiment we used strains LTY564 (*pGAL1-10 PAC2-His$_6$-HA CEN LYS2, YCpGAL*), LTY566 (*pGAL1-10 PAC2-His$_6$-HA CEN LYS2, pGAL1-10 CIN1-HA CEN URA3*), JFY478 (YCpGAL, *pGAL1-10 PAC2-HA CEN LYS2*), and JFY481 (*pGAL1-10 CIN1-HA CEN URA3, pGAL1-10 PAC2-HA CEN LYS2*).

RESULTS

Mutations in the *CIN* genes are sensitive to Rbl2p overproduction: We searched for mutations that affect the formation of tubulin heterodimers. Our strategy was based on the observation that overexpression of the β-tubulin binding protein Rbl2p kills *tub1-724* cells expressing an α-tubulin with relatively weak affinity for β-tubulin (Archer *et al.* 1995; Vega *et al.* 1998). This lethal interaction is probably explained by formation of the Rbl2p-β-tubulin complex at the expense of heterodimer containing the mutant α-tubulin protein. We reasoned that other mutations affecting heterodimer stability or formation might be similarly affected by excess Rbl2p.

We mutagenized wild-type haploid cells containing a *GAL-RBL2* plasmid (pA5) to find mutants that could not survive when *RBL2* was overexpressed (see materials and methods). This screen identified one mutant that was unable to live when overproducing Rbl2p. The mutant strain is extremely sensitive to benomyl and cold sensitive for growth, two properties associated with many mutations affecting microtubules in yeast.

By several criteria, we demonstrated that this mutation is an allele of *CIN1*. A library plasmid containing the entire *CIN1* open reading frame rescued both the benomyl supersensitivity of the mutant and the lethality upon *RBL2* overexpression. To confirm that loss of *cin1* function is sufficient to confer these phenotypes, we deleted the entire open reading frame of *CIN1* by integrative transformation in a wild-type strain and tested the effect of *RBL2* overexpression. As shown in Figure 1, *cin1*Δ cells overproducing Rbl2p start to lose viability ~4 hr after induction and, after ~12 hr, <0.1% of the cells are viable. Finally, we confirmed that the original mutation is indeed an allele of *CIN1* by both complementation and linkage analysis (data not shown) using a *cin1* null allele (Stearns *et al.* 1990).

The *S. cerevisiae* genes *CIN2* and *CIN4* have functions similar to that of *CIN1*, and their products are thought to act as components of a complex or in a common pathway (Stearns *et al.* 1990). We tested whether that

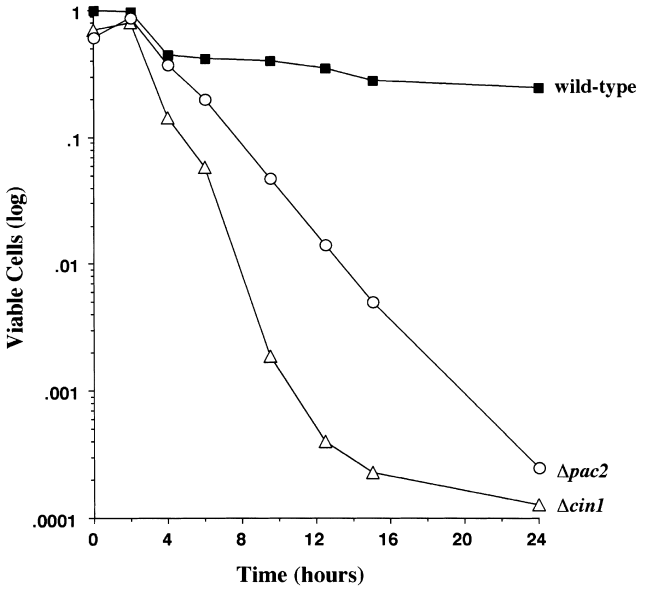

FIGURE 1.—The lethality of *RBL2* overexpression is enhanced in *CIN1* and *PAC2* nulls. *cin1*Δ (△), *pac2*Δ (○), and wild-type strains (■) containing a *GAL-RBL2* plasmid were grown overnight in selective noninducing media. At $t = 0$ hr, Rbl2p overproduction was induced by addition of galactose to 2%. Cell viability is determined as the percentage of cells able to form colonies on glucose plates. Results shown are representative of three independent trials.

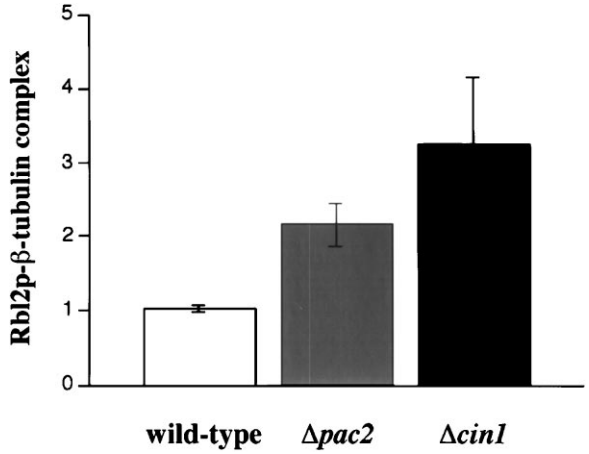

FIGURE 2.—Enhanced formation of Rlb2p-β-tubulin complex in *CIN1* and *PAC2* nulls. Protein extracts from *cin1*Δ, *pac2*Δ, and wild-type strains containing a *GAL-RBL2-HIS*$_6$ plasmid were obtained from cells grown 3 hr in selective inducing media. The tagged Rbl2p and bound proteins were purified using nickel-agarose. Nickel eluates were analyzed by immunoblotting. The Rbl2p and β-tubulin signals were quantitated by densitometry and normalized to Rbl2p signal. The values are expressed as fold increase above the wild-type control. Error bars represent the standard deviation of three independent trials.

functional relationship extends to interaction with Rbl2p. Overexpression of Rbl2p in *cin2* and *cin4* null strains also causes loss of viability (data not shown). The results suggest that the common microtubule-related functions of these three *CIN* genes are affected by Rbl2p levels. However, Cin2p or Cin4p homologues have not been identified in the *in vitro* heterodimerization reaction.

We also tested for interactions between Rbl2p levels and the absence of the two other yeast homologues of the *in vitro* factors, Pac2p/cofactor E and Alf1p/cofactor B. Pac2p/cofactor E binds to α-tubulin *in vitro* (TIAN *et al.* 1997), *in vivo* (VEGA *et al.* 1998), and by two-hybrid assay (FEIERBACH *et al.* 1999). In the *in vitro* assay, it participates in a quaternary complex with both tubulin polypeptides and Cin1p/cofactor D, which dissociates in the presence of cofactor C to release heterodimer. As shown in Figure 1, overexpression of Rbl2p in cells lacking Pac2p causes rapid loss of viability, at a rate comparable to that of cells lacking Cin1p. In contrast, there is no effect of excess Rbl2p in cells that lack Alf1p/cofactor B (data not shown).

Rbl2p-β-tubulin formation and microtubule depolymerization in *pac2* and *cin1* cells overexpressing Rbl2p: The lethality of excess Rbl2p in *tub1-724* cells is accompanied by enhanced formation of Rbl2p-β-tubulin complex and loss of assembled microtubules (VEGA *et al.* 1998). We tested whether the lethality of excess Rbl2p in *cin1*Δ and *pac2*Δ cells has the same properties.

To assay the levels of Rbl2p-β-tubulin complex, extracts were prepared from cells transformed with a plasmid encoding His$_6$-Rbl2p under the control of the galactose promoter and grown for 4 hr in inducing medium. His$_6$-Rbl2p and bound proteins were specifically purified on nickel-agarose beads. The amount of β-tubulin associated with the His$_6$-Rbl2p fraction was two- to threefold higher in *pac2*Δ and *cin1*Δ cells than in wild-type cells (Figure 2). For comparison, the formation of His$_6$-Rbl2p-β-tubulin complex in *tub1-724* cells under the same conditions is fivefold greater than that in wild type (VEGA *et al.* 1998). There is no significant binding of α-tubulin to His$_6$-Rbl2p in any of the strains (data not shown).

The effect on microtubule structures was assayed by immunofluorescence after 3 hr of Rbl2p overexpression (Figure 3). At this time, 78% of wild-type cells have intranuclear microtubules, 18% show a dot representing the spindle pole body, and 4% have no detectable staining. In contrast, only 25% of either *cin1*Δ or *pac2*Δ cells overexpressing Rbl2p have short or long spindles; the remainder have either single dots (28% for *cin1*Δ; 38% for *pac2*Δ) or no staining at all.

Synthetic interactions of *cin1*Δ and *pac2*Δ: Previous work established that *RBL2* is essential in a small subset of specific α-tubulin mutants (ARCHER *et al.* 1995). To test if *cin1* and *pac2* have similar interactions, we constructed strains bearing null alleles of each of those genes and a plasmid expressing wild-type α-tubulin (marked with *URA3*) as their major source of α-tubulin. We transformed those strains with each of 12 different

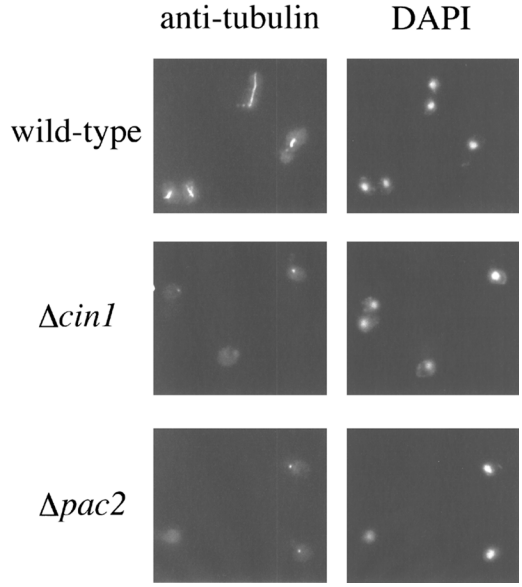

FIGURE 3.—Microtubule staining in *cin1*Δ and *pac2*Δ cells overexpressing *RBL2*. After 3 hr of *RBL2* overexpression, *cin1*Δ (two independent trials), *pac2*Δ (four independent trials), and wild-type cells were processed for tubulin immunofluorescence using an anti-β-tubulin antibody and with DAPI to stain nuclei.

FIGURE 4.—Cells lacking both *cin1* and *rbl2* are viable. *cin1*Δ Δ*rbl2* haploid cells bearing *URA3-CEN* plasmids that express either *CIN1* (JFY232) or *RBL2* (JFY234) were plated to medium containing 5-FOA. Only cells that can lose the *URA3-CEN* plasmid can grow on this medium, which selects for cells that can lose the plasmid. Both double-mutant strains grew as well on this medium as the control, a wild-type strain (FSY183) containing the YCp50 *CEN-URA3* plasmid.

tub1 mutants and then used the drug 5-FOA to identify those mutants that could not lose the plasmid carrying wild-type α-tubulin. We found that 5 of the 12 α-tubulin mutations tested do not support growth of *pac2*Δ and *cin1*Δ cells (Table 2). Significantly, 4 of these 5 mutants are also synthetically lethal with *rbl2*Δ; among these is *tub1-724*, which encodes an α-tubulin defective in β-tubulin binding (VEGA *et al.* 1998). In contrast, several other α-tubulin alleles do not interact with any of these 3 deletion mutants. The results suggest that *PAC2*, *CIN1*, and *RBL2* affect related functions.

As noted above, none of these three genes is essential. In addition, cells containing each of the pairwise combinations of the three mutations are viable. FEIERBACH *et al.* (1999) reported that they could not recover *cin1*Δ, *rbl2*Δ cells. Since Cin1p/cofactor D and Rbl2p/cofactor A perform partially redundant functions in the *in vitro* assay, together they might define an essential function *in vivo*. However, as shown in Figure 4, haploid strains deleted for both *cin1* and *rbl2* and containing a plasmid with a genomic copy of either *CIN1* or *RBL2* marked with the *URA3* gene grow in the presence of the drug 5-FOA, which selects for loss of the covering plasmid. We can also recover double mutants without a plasmid containing either wild-type gene from sporulated cells (data not shown).

TABLE 2

α-Tubulin alleles synthetic lethal with nulls in RBL2, C1N1, and PAC2

	Viability		
	*rb2*Δ	*cin1*Δ	*pac2*Δ
tub1-724, -728, -738, -759	−	−	−
tub1-735	ND	−	−
tub1-704, -714, -744, -750	+	+	+
tub1-727, -730, -733, -741, -746, -758	ND	+	+

ND, not determined.

The effect of *CIN* gene overexpression on *tub1-724* mutant phenotypes: We previously demonstrated that the conditional lethality in strains expressing *tub1-724* is a consequence of dissociation of the unstable tubulin heterodimer to produce free β-tubulin (VEGA *et al.* 1998; see DISCUSSION). Consistent with that conclusion, high levels of either Rbl2p/cofactor A or Pac2p/cofactor E kill *tub1-724* haploid cells (ARCHER *et al.* 1995; VEGA *et al.* 1998).

Overexpression of *CIN* genes in *tub1-724* cells yields a dramatically different result. We introduced a plasmid containing *CIN1* under control of the *GAL* promoter into the *tub1-724* mutant strain and monitored cell growth under various conditions. Excess Cin1p suppresses the growth defect of *tub1-724* cells at 25°, a semipermissive temperature for this mutant (Figure 5A). In addition, excess Cin1p suppresses the lethality of *PAC2* overexpression in these cells (Figure 5B). This effect of extra Cin1p is specific for the *tub1-724* mutant; it has no effect on the cold-sensitive growth of the other α-tubulin alleles listed in Table 2.

In principle, a possible explanation for this suppression is that Cin1p sequesters free β-tubulin and so interferes with its toxicity. Indeed, Cin1p/cofactor D does bind to β-tubulin *in vitro* (TIAN *et al.* 1996) and *in vivo* (see below). Contrary to that hypothesis, however, overexpression of *CIN1* does not rescue cells from excess β-tubulin in two other contexts. First, overexpression of

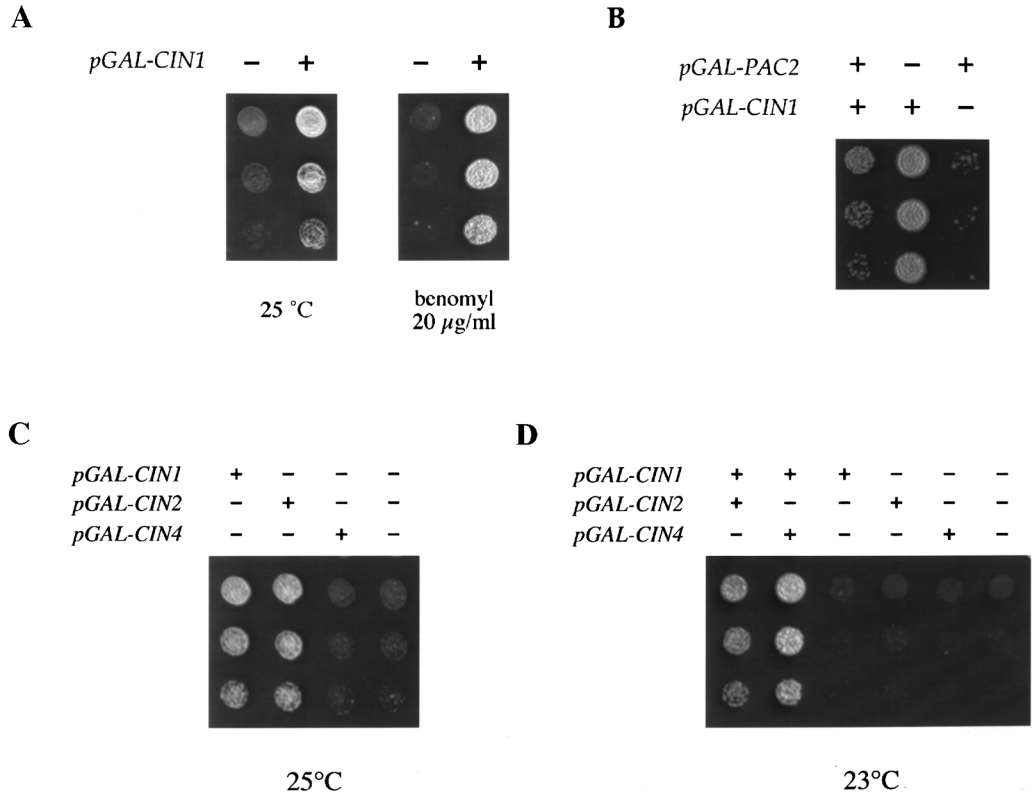

FIGURE 5.—Overexpression of *CIN1* is able to rescue conditional phenotypes of the *tub1-724* mutant. (A) Stationary phase cultures of *tub1-724* mutant cells (FSY157) containing either *GAL-CIN1* or a YCpGAL control plasmid were serially diluted (one-sixth dilutions for the 25° plate, one-fourth for the benomyl plate), spotted to selective galactose plates with or without 20 μg/ml benomyl, and incubated at 25°. (B) Stationary phase cultures of *tub1-724* mutant cells (FSY157), each containing two plasmids, were serially diluted (one-fourth dilutions) and spotted to selective galactose plates. The plasmids were as follows: *GAL-CIN1* (pJF17) and *GAL-PAC2* (pJF16) (left column), *GAL-CIN1* and pRS317 (middle column), and *GAL-PAC2* and YCpGAL (right column). (C) Stationary phase cultures of *tub1-724* mutant strains containing *GAL-CIN1* plus the pRS313 control (first row), *GAL-CIN2* or *GAL-CIN4* plus the YCpGAL control plasmid (second and third rows), or control plasmid alone (fourth row) were serially diluted (one-fourth dilutions) and plated to selective galactose plates at 25°. (D) Stationary phase cultures of *tub1-724* mutant strains containing *GAL-CIN1* plus *GAL-CIN2* or *GAL-CIN4*, or each of the individual plasmids, were serially diluted (one-fourth dilutions) and plated to selective galactose plates at 23°.

Cin1p, unlike overexpression of either Rbl2p or α-tubulin, does not rescue excess β-tubulin lethality. For example, the plating efficiency on galactose of cells containing a single *GAL-TUB2* gene is 0.01% in the presence or absence of a *GAL-CIN1* plasmid (data not shown), but increases to 70% when either *GAL-RBL2* or *GAL-TUB1* are present (ARCHER *et al.* 1995). Second, overexpression of *CIN1* does not rescue the benomyl supersensitivity of a *tub3*Δ strain (HOYT *et al.* 1997 and J. FLEMING, unpublished results), which has a modest constitutive excess of β-tubulin. Significantly, the benomyl and cold sensitivities of *tub3*Δ cells are markedly less severe than those of *tub1-724* cells, suggesting that the levels of excess β-tubulin are higher in the latter strain. Therefore, the differential suppression by Cin1p overproduction in these two mutants is not a consequence of the relative levels of free β-tubulin. Instead, the distinction between the one situation in which excess Cin1p does suppress and the two in which it does not can be rationalized on the basis of levels of α-tubulin. *tub1-724* cells contain stoichiometric α- and β-tubulin, while both *TUB2* overexpressers and *tub3*Δ cells contain an excess of β-tubulin.

Since *CIN2* and *CIN4* have properties similar to *CIN1* in other assays, we tested them for interaction with *tub1-724*. Overexpression of *CIN1*, *CIN2* but not *CIN4* partially rescues the *tub1-724* growth phenotype at 25° (Figure 5C, lanes 2 and 3). At 23°, none of the overexpressed *CIN* genes has a significant effect on *tub1-724* growth (Figure 5D). However, overexpression of *CIN1* in combination with either *CIN2* or *CIN4* both significantly enhance cell growth. The results suggest that, under restrictive conditions, the expression levels of each of the *CIN* genes can be limiting for growth.

Physical interactions of Cin1p: To assay yeast cell extracts for the protein complexes suggested by the *in vivo* results above and the *in vitro* data, we made a HA-His_6-tagged version of Cin1p behind control of the *GAL* promoter (pJF15). Figure 6A shows that β-tubulin but not α-tubulin specifically copurifies with the tagged Cin1p (five independent trials). In contrast, there is no detectable enrichment of α-tubulin among the proteins eluted with Cin1p. Formation of the Cin1p-β-tubulin-containing complex is independent of Pac2p (data not shown). This result suggests that Cin1p can bind directly or indirectly to β-tubulin but not α-tubulin *in vivo*, similar to Rbl2p. The significance of this complex is analyzed in DISCUSSION. To assay for a complex between Cin1p and Pac2p, we overexpressed HA-tagged versions of

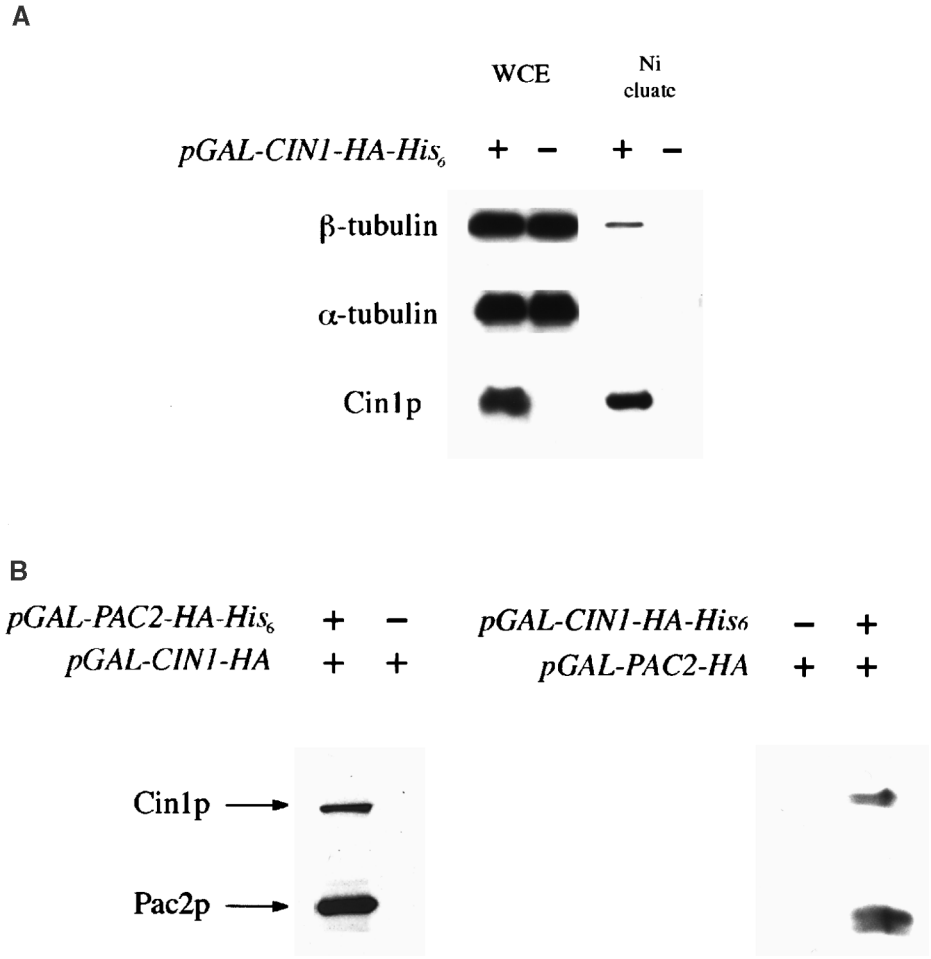

FIGURE 6.—Interactions among Cin1p, Pac2p, and tubulin. (A) Extracts from wild-type strains containing either *GAL-CIN1-HA-His*$_6$ or YCpGAL control plasmid were obtained after 4 hr of growth in inducing media. The whole cell extract (WCE) was probed directly for the tubulin polypeptides and Cin1p, or applied to Ni-NTA columns (see MATERIALS AND METHODS) to purify the tagged Cin1p and proteins bound to it. The immunoblots were probed with antibodies against α-tubulin, β-tubulin, and the HA epitope tag (for HA-Cin1p). The data demonstrate that the α-tubulin and β-tubulin signals are comparable in the whole cell extracts, but that β-tubulin binds preferentially to Cin1p. (B) Cin1p and Pac2p bind *in vivo*. Cells containing the plasmids indicated were grown on galactose for 4 hr, and extracts were incubated with Ni-NTA beads, eluted, and assayed by immunoblotting. Cin1p and Pac2p coelute from beads when either of the proteins bears a His-6 tag. The results shown are representative of nine independent trials using His-tagged Pac2p and two independent trials using His-tagged Cin1p.

both proteins. Using affinity chromatography, we show that Pac2p copurifies with the His$_6$-tagged Cin1p and, conversely, that Cin1p specifically copurifies with a His$_6$-tagged version of Pac2p (Figure 6B).

DISCUSSION

The data presented here identify genes that affect tubulin dimer formation *in vivo*. Previous work demonstrated that Rbl2p can compete with α-tubulin for binding to β-tubulin and so disrupt the heterodimer. Therefore, an α-tubulin mutant protein (Tub1-724p) that has reduced affinity for β-tubulin is supersensitive to Rbl2p overexpression. Here we show that mutations in four nontubulin genes—*CIN1*, *2*, *4*, and *PAC2*—similarly enhance the lethality of excess Rbl2p. Their function is identified by their interaction with the mutant α-tubulin protein, Tub1-724p. The conditional lethality of that mutant is due to the dissociation of tubulin heterodimer to produce free toxic β-tubulin (VEGA *et al.* 1998 and see below). Both Pac2p and Cin1p are required in *tub1-724* cells, and overexpression of the *CIN* genes suppresses *tub1-724* phenotypes. The results provide evidence that the products of these four genes participate in tubulin heterodimerization *in vivo*. Below, we discuss the activities of these different genes *in vivo* and how they correlate with the properties of their mammalian homologues in *in vitro* assays. We consider the evidence supporting their roles in *de novo* formation of heterodimer, and we propose an alternative, salvage pathway that acts on the products of dissociated heterodimer to protect the cell against accumulation of toxic free β-tubulin.

Consequences of Rbl2p overproduction in *cin1* and *pac2* nulls: We report three consequences of overexpressing *RBL2* in *cin1* and *pac2* mutant cells: loss of cell viability, loss of assembled microtubules, and enhanced formation of Rbl2-β-tubulin complex. These three phenotypes also occur when *RBL2* is overexpressed in *tub1-724* cells (ARCHER *et al.* 1995; VEGA *et al.* 1998). In the latter instance, the phenotypes are all readily interpreted given that the mutant α-tubulin forms a heterodimer that is less stable than wild type. However, as Cin proteins and Pac2p are not stoichiometric components of the tubulin heterodimer, they obviously do not directly stabilize the heterodimer. An alternative explanation is that these proteins act to oppose the destabilization of heterodimer by facilitating heterodimer formation. Although not normally essential, they become essential (STEARNS *et al.* 1990; HOYT *et al.* 1997; Figure 5 above)

when microtubule assembly is inhibited by depolymerizing drugs or growth at low temperature and the level of heterodimer is correspondingly increased.

Effects of Cin1p and Pac2p levels in tubulin mutants: Null alleles of *cin1*, *pac2*, and *rbl2* are lethal in combination with the same four cold-sensitive α-tubulin mutants, suggesting that the mutants all affect a related function. Although those four mutants all arrest with no microtubules (Schatz *et al.* 1988), several other mutants with the same phenotype are not lethal in combination with *cin1*Δ, *pac2*Δ, or *rbl2*Δ.

The molecular defect of one of the mutants is well understood: the α-tubulin in *tub1-724* cells forms a less stable heterodimer (Vega *et al.* 1998). Significantly, *tub1-724* is semidominant: *TUB1/tub1-724* heterozygotes show enhanced sensitivity to low temperature and microtubule depolymerizing drugs. Although overexpression of the β-tubulin binding protein Rbl2p is lethal in *tub1-724* cells, it suppresses the phenotypes of the heterozygote. This result demonstrates that the phenotypes conferred by the Tub1-724p mutant α-tubulin in the heterozygote can all be ascribed to the free β-tubulin released by dissociation of the mutant heterodimer. This finding is consistent with, but does not prove, the hypothesis that the conditional defect in *tub1-724* haploid cells also is a consequence of heterodimer dissociation and the formation of free β-tubulin. This mutation may confer other defects in microtubule morphogenesis, but since the release of free β-tubulin is sufficient for cell death, the suppression by excess Cin1p of *tub1-724* haploids means that the excess Cin1p must at least suppress the formation of free β-tubulin or its downstream effects.

This property of *tub1-724* provides insight into the activities of *PAC2* and *CIN1*. The most informative phenotypes are those of *tub1-724* cells overexpressing either Pac2p or Cin1p. Excess Pac2p is lethal. We previously showed that Pac2p is an α-tubulin binding protein (Vega *et al.* 1998); its overexpression in the presence of a weakened heterodimer would be expected to release toxic free β-tubulin.

Similarly, the fact that Cin1p binds β-tubulin (Figure 6A) leads to the expectation that its overexpression would be lethal in *tub1-724* cells, similar to overexpressed Rbl2p. However, we found that excess Cin1p actually suppresses the conditional phenotypes of this mutant α-tubulin. In addition, excess Cin1p does not rescue the phenotypes associated with excess β-tubulin in *tub3*Δ (Hoyt *et al.* 1997) or β-tubulin overproducing strains (data not shown). Taken together, these observations suggest that excess Cin1p does not act like Rbl2p to protect cells from excess β-tubulin. Moreover, the phenotypes of the *tub1-724* mutation are more severe than those of a strain deleted for *tub3*, suggesting that *tub1-724* cells have more free β-tubulin. A distinct difference between these two mutations is that the *tub1-724* cells contain a pool of undimerized α-tubulin stoichiometric with the β-tubulin, while the *tub3*Δ and *GAL-TUB2* strains do not. This distinction may explain why overexpressed Rbl2p but not Cin1p rescues high-level overexpression of β-tubulin. Thus, Cin1p suppressing activity is not explained by its β-tubulin binding activity alone.

Binding partners of Cin1p: The results above also demonstrate physical interactions that are consistent with the functional data and with the *in vitro* data. Fractionation experiments performed using Cin1p and Pac2p have allowed us to characterize their possible interactions *in vivo*. We show that, as for the mammalian cofactor D, β-tubulin but not α-tubulin copurifies with Cin1p when it is overexpressed in wild-type cells. That complex is detected in the presence or absence of Pac2p. In addition, we have also shown that Cin1p and Pac2p associate *in vivo*, an interaction not detected using the two-hybrid assay (Feierbach *et al.* 1999).

Comparison of *in vivo* and *in vitro* properties of tubulin binding proteins: Cowan and colleagues identified purified protein factors that can help catalyze incorporation of β-tubulin and α-tubulin into exogenous heterodimer (Gao *et al.* 1992, 1993; Tian *et al.* 1996, 1997). In that assay system, β-tubulin released from the chaperone is bound by either cofactor A/Rbl2p or cofactor D/Cin1p, and α-tubulin is bound by either cofactor B/Alf1p or cofactor E/Pac2p. The β-tubulin released from cofactor A and the α-tubulin released by cofactor B must bind cofactors D and E, respectively, before they can be incorporated into heterodimer. Cofactor D-β-tubulin and cofactor E-α-tubulin form a quaternary complex. Cofactor C is believed to mediate the release of α-β tubulin heterodimer. It is not known whether these protein cofactors, required under the conditions of this assay, are essential *in vivo* or whether they account for all the activity found in the original extracts.

Four of these mammalian cofactors are homologous to yeast genes: Cofactor D shows 21% identity with Cin1p (Hoyt *et al.* 1997); cofactor E is 30% identical to Pac2p (Hoyt *et al.* 1997); cofactor A is structurally and functionally homologous to Rbl2p (Archer *et al.* 1995); and cofactor B is 32% identical to Alf1p (Tian *et al.* 1996). In the *in vitro* assay, cofactors D and E are essential. However, none of the homologous yeast genes are essential. Several groups also have demonstrated that even several pairwise deletions—*CIN1* and *PAC2* (Hoyt *et al.* 1997 and our unpublished results), *RBL2* and *PAC2* (our unpublished results), and *ALF1* in pairwise combinations with each of the other three genes (Feierbach *et al.* 1999)—do not define an essential function. Also, in contrast to a previous report (Feierbach *et al.* 1999), we find that cells deleted for both *cin1* and *rbl2* are viable (see Figure 4 above). These results argue strongly that this cofactor-dependent pathway is not essential for making tubulin heterodimers *in vivo*. There may be redundant functions in yeast specified by genes as yet undetected.

Several of the functional interactions we detect *in vivo* among these proteins are consistent with this *in vitro* model. However, other results demonstrate significant differences between the *in vivo* and *in vitro* situations. Most important, suppression by excess Cin1p/cofactor D of a mutant α-tubulin with lowered heterodimer stability directly contradicts the *in vitro* model: Cofactor D *in vitro* disrupts the heterodimer to form a cofactor D-β-tubulin complex (Tian *et al.* 1997). Also, Cin1p activity *in vivo* does not require stoichiometric Pac2p: Overexpression of Cin1p alone is sufficient to suppress *tub1-724* and can rescue the benomyl supersensitive phenotype of *pac2*Δ strains (Hoyt *et al.* 1997). These *in vivo* results suggest that Cin1p action is not confined to bringing β-tubulin into a quaternary complex containing Pac2p and α-tubulin, as hypothesized for the *in vitro* action of cofactor D. Finally, unlike the *in vivo* situation, to date no role for Cin2p or Cin4p vertebrate homologues has been reported for the *in vitro* assay.

Significantly, our results demonstrate that the interactions of Rbl2p and Cin1p with β-tubulin *in vivo* must be quite different; excess of the former kills *tub1-724* cells, while the latter suppresses the same mutation. This distinction contrasts with the *in vitro* results suggesting that Rbl2p and Cin1p can bind equivalent forms of β-tubulin.

The *in vivo* data clearly demonstrate that the Cin1p-β-tubulin and Rbl2p-β-tubulin complexes are functionally quite different. For example, excess Rbl2p suppresses the lethality of free β-tubulin, but excess Cin1p does not. Conversely, Cin1p interacts with β-tubulin to promote heterodimer assembly, but Rbl2p does not.

A pathway for rescuing dissociated tubulin heterodimers: The experiments presented here demonstrate that proteins that interact with individual tubulin polypeptides can influence the formation of heterodimer *in vivo*. Especially in the case of Cin1p, the relationship between phenotype and expression levels suggests that this protein acts to promote α-β-tubulin complex formation and not merely by binding reversibly to free β-tubulin. Such an activity will require coupling to a highly exergonic step to make the reaction act as if it were unidirectional. A candidate for that coupling factor is Cin4p: It has a predicted GTP binding motif (Hoyt *et al.* 1997) and it enhances the ability of Cin1p to rescue *tub1-724* cells (Figure 5D).

Since neither *CIN1* nor *PAC2* is essential in otherwise wild-type cells, it is unlikely that they are important in the major pathway of *de novo* heterodimer formation. An alternative possibility is suggested by the fact that they become essential under conditions that would be expected to increase levels of free tubulin polypeptides. First, both are essential in *tub1-724* cells, in which the mutant α-tubulin destabilizes the heterodimer and favors its dissociation. Second, cells deleted for *cin1*, *cin2*, *cin4*, or *pac2* are supersensitive to low temperature and benomyl, two treatments that cause microtubule depolymerization; the increased level of heterodimer produced by microtubule disassembly will in turn generate an increased steady state level of free tubulin polypeptides as a result of dissociation. Third, both *cin1* and *pac2* mutant cells show enhanced formation of Rbl2p-β-tubulin complex upon Rbl2p overexpression. Finally, *pac2* and *cin1* are synthetically lethal with *pac10*, which encodes a nonessential tubulin folding factor (Alvarez *et al.* 1998). Improper folding of α- and β-tubulin could also lead to higher levels of undimerized tubulin polypeptides.

A possible function for these genes is that they facilitate reassociation of tubulin polypeptides to reform heterodimer, thus protecting the cell against free tubulin polypeptides and maintaining required levels of the heterodimer itself. In contrast to the *in vitro* assay for tubulin heterodimer formation from denatured tubulin, in which the mammalian homologues of Cin1p and Pac2p are essential, facilitated heterodimer formation may be only conditionally required *in vivo*. The roles of Pac2p/cofactor E and Cin1p/cofactor D in such a mechanism are consistent with many of their activities in the *in vitro* assay. The data also predict that Cin2p and Cin4p would enhance Cin1p-mediated heterodimer formation in an *in vitro* assay. We cannot test directly whether levels of tubulin heterodimer in *tub1-724* cells change as a function of Cin1p expression, since the mutant heterodimer dissociates under the conditions required for its isolation (Vega *et al.* 1998). However, detailed analysis of other α-tubulin mutants that interact with *CIN1* levels may provide more direct tests of this model.

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