ANALYSIS OF MICROTUBUBULE MORPHOGENESIS IN VIVO

by

Julie Elizabeth Archer

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Signature of Author .....
Department of Biology
August 28, 1996

Certified by .....
Dr. Frank Solomon
Professor, Biology
Thesis Supervisor

Accepted by .....
Dr. Frank Solomon
Professor, Biology
Chair, Departmental Graduate Committee

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ABSTRACT

Microtubule assembly occurs from a pool of α-β-tubulin heterodimers. Heterodimers themselves are the result of an assembly pathway involving folding and dimerization. We have characterized Rbl2p, a yeast protein that binds to a monomeric form of β-tubulin.

We identified *RBL2* in a screen for yeast genes that when overexpressed can rescue cells from the lethality caused by an excess of β-tubulin. *RBL2* is not essential for vegetative growth, but changes in its level affect microtubule pathways and meiosis. *RBL2* over-expression, like the over-expression of α-tubulin, confers resistance to benomyl. Both the deletion and the over-expression of *RBL2* are synthetically lethal with specific mutant alleles of α-tubulin. Rbl2p is structurally and functionally homologous to murine cofactor A. Cofactor A was identified as a polypeptide essential for tubulin folding and dimerization in an *in vitro* assay. Our results suggest that Rbl2p interacts with monomeric ß-tubulin as a distinct step in an assembly pathway, independent of the chaperonin-mediated folding. Furthermore, Rbl2p appears to recognize a nearly native form of β-tubulin, a conformation very similar to that which binds α-tubulin. Finally, we have identified an allele of a yeast gene, *rks1-1*, that genetically interacts with *RBL2* and exhibits a microtubule phenotype. The product of a suppressor of *rks1-1*, *SUP1*, physically interacts with Rbl2p and also exhibits microtubule phenotypes. Our results suggest that we have begun to define a group of microtubule assembly proteins that may serve an analogous role to scaffolding proteins in other morphogenetic pathways - participating in intermediates but not present in the final product.

Thesis Supervisor: Frank Solomon
Title: Professor of Biology

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CHAPTER 1

INTRODUCTION

DETERMINANTS THAT SPECIFY MICROTUBULE FORM

Microtubule assembly and organization is subject to regulation by non-tubulin proteins. From the same building blocks, the pattern of microtubule geometries can vary considerably in different cells types and within the same cell at different stages in the cell cycle. Although pure tubulin is sufficient to recreate microtubules *in vitro*, other factors are required to account for the range of shapes and the dynamic properties of microtubules *in vivo*.

Microtubule associated proteins

Historically, it has been logical to focus on microtubule associated proteins, or MAPs, as likely candidates for factors affecting microtubule stability. That is, bovine brain proteins that co-purified with tubulin during rounds of temperature induced assembly and disassembly have been named and numbered as MAPs (Weingarten et al., 1975). These include MAP1A (Bloom et al., 1984), MAP1B (Noble et al., 1989), MAP2 (Shiomura and Hirokawa, 1987), MAP4 (Parysek et al., 1984), and tau (Drubin and Kirschner, 1986). Binding of MAPs to microtubules in vitro or after transfections in vivo seems to promote bundling and inhibit depolymeration of microtubules (Cleveland et al., 1977; Sandoval and Vandekerckhove, 1981; Knops et al., 1991; Leclerc et al., 1996). Results of antisense experiments are also consistent with a role of MAP2 and tau in stabilizing microtubules (Dinsmore and Solomon, 1991; Caceres and Kosik, 1990). These MAPs (with the exception of MAP4) are expressed in differentiated neuronal cells, so they do not appear to play a role in mitosis. E-MAP-115 is expressed in dividing cells and appears to bind to and stabilize microtubules in a cell cycle dependent fashion (Masson and Kreis, 1995).

The tubulin of Saccharomyces cerevisiae is unable to cycle *in vitro*, so the approach to obtaining MAPs is different. Curiously, none of the mammalian MAPs have obvious homologs in yeast. Therefore, candidates must be identified genetically as affecting microtubule function and then assayed for *in vivo* localization. Genes identified in this manner that co-localize with microtubules include *BIK1*, and *STU1*. *BIK1* was identified in a screen for karyogamy defects (Berlin et al., 1990). Karyogamy, or nuclear fusion is one of the events identified using microtubule depolymerizing drugs as events that require microtubules (Jacobs et al., 1988). In addition Δ*bik1* cells exhibit defects in chromosome segregation and nuclear migration. Bik1p localizes with the nuclear spindle (see below). *STU1* was identified as a suppressor of a ß-tubulin mutation (Pasqualone and Huffaker, 1994). *STU1* genetically interacts with *TUB1* (encoding α-tubulin (Schatz et al., 1986)) and *TUB2* (encoding β-tubulin (Neff et al., 1983)). It is an essential gene whose product localizes to the mitotic spindle. Kem1p/Sep1 is also a candidate MAP. *KEM1* was isolated in a screen for mutations that enhanced the nuclear fusion defect of *kar1-1* (Kim et al., 1990). *kem1* strains exhibit microtubule defects such as chromosome instability and supersensitivity to benomyl. Kem1p/Sep1p promotes polymerization of microtubules and co-sediments(Interthat et al., 1995), but localization studies have not yet been reported. Unlike the case of mammalian MAPs, the yeast MAPs identified so far localize to mitotic microtubules not developmentally specialized microtubules such as the microtubules involved in nuclear fusion. An overexpressed KAR1-βgalactosidase fusion protein localized to these structures (Vallen et al., 1992), but the endogenous protein does not (Spang et al., 1995).

Microtubule nucleation

In addition to stabilizing the length of microtubules, proteins can affect microtubule distribution by directly or indirectly controlling microtubule nucleation. γ-tubulin is a strong candidate for a component of the nucleation site. The γ-tubulin gene was first identified as a suppressor of a ß-tubulin mutant in Aspergillis nidulans (Oakley and Oakley, 1989) and localizes to the microtubule organizing center (MTOC) in many organisms (Oakley et al., 1990; Stearns et al., 1991; Zheng, et al., 1991). γ-tubulin purifies in a soluble form as a ring (Zheng et al., 1995) and this same complex appears to be intact within MTOCs (Moritz et al., 1995). This complex is sufficient to promote microtubule nucleation *in vitro* (Zheng et al., 1995). Antibody injection and depletion experiments are consistent with a requirement for γ-tubulin in microtubule nucleation (Stearns and Kirschner, 1994), but results with conditional mutants in *S. cerevisiae* are not.

Other MTOC components also exhibit microtubule phenotypes. In yeast, Kar1p (Rose and Fink, 1987; Spang et al., 1995), Cik1p (Page and Snyder, 1992), Cdc31p (Baum et al., 1986), Spc42p (Donaldson and Kilmartin, 1996), Spc110p (Kilmartin et al., 1993; Geiser et al., 1993) and calmodulin (Geiser, et al., 1993) all localize to spindle pole bodies (SPB, the yeast MTOC) and mutants show a variety of microtubule defects (Conde and Fink, 1976) (Stirling et al., 1994). These proteins appear to play roles in SPB duplication or structure, so they are probably only indirectly responsible for microtubule assembly and stability. Similarly, the injection of antibodies directed against mammalian proteins that localize to the MTOC, such as pericentrin (Doxsey et al., 1994) and NuMA (Gaglio et al., 1995; Yang and Snyder, 1992),

leads to defects in microtubules. Although this assay is on a shorter time course which could prevent the accumulation of indirect defects, the presence of the antibodies themselves raises the possibility of steric hindrance close to the actual site of nucleation.

In addition, Surridge and Burns (1991) identified an activity that appears to inhibit microtubule assembly through a mechanism of decreasing nucleation events, rather than limiting the final extent of assembly.

Microtubule dynamics

Microtubule mass and geometry is affected by specific dynamic parameters (Mitchison and Kirschner, 1984), namely rate of polymerization, rate of depolymerization, frequency of shift from growing to shrinking (catastrophe), and frequency of shift from shrinking to growing (rescue) (Belmont et al., 1990; Verde et al., 1990; Glicksman et al., 1992; Dreschel et al., 1992; Vasques, et al., 1994). Potential sites of control include GTP hydrolysis and GDP-GTP exchange, because GTP-bound tubulin is assembly competent and a GTP cap is postulated to be necessary for microtubule stability (Caplow and Shanks, 1996). Recently, the Mitchison lab has identified two proteins that appear to affect frequency of catastrophe. As one of them, XKCM1, has sequence homology to kinesin (Walczak et al., 1996) while the other, oncoprotein 18/stathmin, is a small phosphoprotein (Belmont and Mitchison, 1996), they potentially have quite different mechanisms of action. XKCM1 localizes to the ends of spindle microtubules. Depletion of XKCM1 results in a decrease in catastrophe and longer microtubules (Walczak et al., 1996). Oncoprotein 18/stathmin is a tubulin dimer binding protein. It was purified

by its microtubule-destabilizing activity and its depletion leads to an increase in aster size in Xenopus extracts (Belmont and Mitchison, 1996).

Spindle assembly

The assembly of a spindle occurs with a cell cycle-dependent switch in microtubule dynamics coordinated with a spatial design. Furthermore, the spindle is not a differentiated, stable cytoskeletal structure but rather a transient one that must rapidly reorganize during stages of mitosis and ultimately, disassemble. Cell cycle phosphorylation events at the the SPB and kinetochores may be significant for these processes (Centonze and Borisy, 1990; Engle et al., 1988; Campbell and Gorbsky, 1995).

The production of a metaphase spindle in yeast depends on the motor proteins *CIN8*, *KIP1* (Saunders and Hoyt, 1992) and *DHC1/DYN1* (Saunders et al., 1995). In Xenopus egg extracts, the kinesin-like Eg5 may influence spindle organization, because depletion of this protein results in decreased assembly (Sawin et al., 1992). The spindle may be the balanced sum of forces acting in the nucleus to push SPBs apart and to pull SPBs together, as well as force mediated through cytoplasmic microtubules tugging on the SPBs (Saunders and Hoyt, 1992).

Anaphase B may be triggered by the degradation of factors that maintain the delicate balance (Holloway et al., 1993; Surana et al., 1993). Such factors may act as "glue" proteins between sister chromatids or as clamp proteins on the microtubule apparatus itself. Candidates for these roles include CP60 (Kellogg et al., 1995), *ASE1* (Pellman et al., 1995) and *PDS1* (Yamamoto et al., 1996). CP60 was isolated through its binding to CP190, and both these

Drosophila proteins are retained on microtubule affinity columns. CP60 localizes to centrosomes until the end of mitosis, when the protein disappears (Kellogg et al., 1995). It contains a putative"destruction box" and so is a candidate substrate for specific proteolysis. *ASE1* was isolated as a mutation that is synthetic lethal with *BIK1*. Ase1p localizes to the spindle midzone until the end of mitosis (Pellman et al., 1995). *PDS1* was isolated as a mutation that exhibited precocious dissociation of sister chromatids and enhanced lethality in the presence of nocodazole, a microtubule depolymerizing drug. *pds1* mutations relieve the arrest caused by mutations in components of the anaphase degradation machinery (APC - *cdc16*, *cdc23*, *cdc27*)(Yamamoto et al., 1996). This result suggests that Pds1p may be a substrate of APC that must be degraded for anaphase to proceed. The generation of non-degradable forms of CP60, Ase1p and Pds1p will provide further evidence regarding the role of their degradation in mitotic progression. One possible mechanism for spindle disassembly is microtubule severing. Katanin is a protein from sea urchin eggs that severs microtubules leading to depolymerization in a ATP dependent reaction (McNally and Vale, 1993).

The proper timing of assembly and disassembly events relies on communication between the participants. Checkpoint genes were first defined as acting outside the processes themselves, performing roles that would be not essential during correctly orchestrated events but only during error (Weinert and Hartwell, 1988; reviewed in Murray, 1994). Recent evidence is consistent with the model that at least some checkpoint genes are intrinsic components of the process itself (Lydall and Weinert, 1995; Al-Khodairy and Carr, 1992). The *BUB* and *MAD* genes were isolated as mutants that were unable to respond properly by arresting when faced with

microtubule defects caused by the drugs benomyl or nocodazole (Hoyt et al., 1991; Li and Murray, 1991). *BUB*s and *MAD*s may participate in a response to unattached kinetochores rather than to microtubule disassembly per se (Wang and Burke, 1995; Wells and Murray, 1996).

There are many ways to affect the assembly and organization of microtubules. In all cases discussed above, the apparent substrate is tubulin dimer or microtubule polymer. However, dimer itself is subject to an assembly pathway that will be discussed in the next section.

TUBULIN FOLDING AND DIMERIZATION

The pathway leading to assembly competent tubulin heterodimer is just beginning to be elucidated. The rough landmarks are ribosome to chaperone to dimer. As is the case for other multi-subunit complexes, there may be additional accessory proteins that play a role in either preventing aggregation or promoting association of subunit before the mature quaternary structure is formed.

Folding by the TCP-1 family

TCP-1 is the locus in mouse that encodes the tailless complex polypeptide 1. By sequence it is related to the archaebacteria chaperonin TF55 (Trent et al., 1991) and the chaperonin hsp60 (Ahmad and Gupta, 1990) and is highly related to a group of at least eight eukaryotic proteins (Ahmad and Gupta, 1990). The TCP1-like family members form a heterooligomeric complex of two stacked rings of eight polypeptides (Lewis et al., 1992; Frydman et al., 1992; Rommelaere et al., 1993). This structure is similar to that of GroEL, the proteasome, and other protein processing complexes (reviewed in Horwich and Willison, 1993).

There is evidence that TCP-1 is important for tubulin folding and that tubulin is one of a small number of TCP-1 substrates. The understanding of this special relationship came about through analysis of both partners. It became clear that α- and β-tubulin require assistance in folding because attempts to produce them in E. coli failed (Zabala and Cowan, 1992; Gao et al., 1993). It also turns out that while TCP-1 genes are essential in yeast, conditional mutants exhibit defects in microtubules and microfilaments (Ursic and

Culbertson, 1991; Ursic et al., 1994; Miklos et al., 1994; Chen et al., 1994; Vinh and Drubin, 1994). Putting these observations together, several groups have performed in vitro assays to demonstrate that the TCP-1 complex can play a role in the folding of α- and β-tubulin as well as actin, γ-tubulin and a few other proteins (Yaffe et al., 1992; Gao et al., 1992; Gao et al., 1993; Melki et al., 1993; Melki and Cowan, 1994; Campo et al., 1994). There is specificity in the other direction as well; tubulin and actin seem to fold in the presence of the TCP-1 complex, but not other chaperonins (GroEL or mitochondrial chaperonin), even though they are able to bind to all three (Tian et al., 1995). There is some evidence that this folding reaction occurs in vivo, because after isolation of pulse-labeled protein from animal cells, tubulin and actin are present in large complexes that contain TCP1 (Sternlicht et al., 1993). At least some of the TCP-1 in HeLa cells localizes to the centrosome and antibodies directed against TCP-1 block growth of microtubules(Brown et al., 1996). These results raise the possibility that TCP-1 may not just act in the cytoplasm but could play at role at the MTOC, such as shuttling dimer to sites of nucleation or binding γ-tubulin until heterodimer displaces it.

It turns out that TCP-1 is sufficient to fold actin and γ-tubulin in *in vitro* assays, but additional cofactors are required for α- and β-tubulin (Gao et al., 1993; Gao et al., 1994; Campo et al., 1994). The assay in this case is incorporation into heterodimer because α- and β-tubulin appear not to exist as monomeric species, for example by native gel analysis. Although the crystal structures are not solved for α- and β-tubulin, it is likely that quasi-native structure leaves hydrophobic residues exposed. These residues would usually be buried within dimer, but in monomeric subunits would promote aggregation. Therefore, the "insufficiency" of TCP-1 to fold α- and β-tubulin

must be tempered by the realization that it might be maintainance of native form that requires additional cofactors.

Specifically, cofactors A and B were required in addition to the TCP1-complex for denatured, recombinant α- and β-tubulin to obtain competency for exchange into exogenous dimer. Cofactor A alone was sufficient for the appearance on a native gel of a form of β-tubulin that ran as monomer, but this β-tubulin was unable to exchange into dimer. We identified a gene in Saccharomyces cerevisiae, *RBL2*, that encodes a homolog to cofactor A (see Chapter 2; Archer et al., 1995). Levels of Rbl2p are important for microtubule function and Rbl2p binds to β-tubulin. The latter result probably explains the ability of cofactor A to stabilize monomeric β-tubulin in the in vitro assay. However, *RBL2* is not an essential gene. Recently, Cowan and coworkers have reported the identification of a new series of murine cofactors required to produce exchange competent β-tubulin (Tian et al., 1996). They now concur that cofactor A is not essential for β-tubulin folding and dimerization. They identified a pathway of cofactors D, E and C. Their model is that cofactor D forms a complex with β-tubulin, then the binding of cofactor E leads to the release of β-tubulin. Finally, cofactor C performs some last step allowing exchange into dimer. Cofactor D is 21% identical to the yeast gene *CIN1*, cofactor E is 30% identical to the yeast gene *PAC2*, and cofactor C is not homologous to any gene of known function. *CIN1,2*, and *4* were identified with chromosome instability defects (Hoyt et al., 1990) and with sensitivity to benomyl (Stearns et al., 1990), a microtubule depolymerizing drug. We observe synthetic lethality of cin1,2 and 4 alleles with *RBL2* overexpression (J. Fleming and F. Solomon, unpublished observations). *PAC2* was identified as synthetically lethal with *CIN8*.

Assembly of complexes

Just as some proteins can fold spontaneously whereas others require the assistance of chaperones, some multi-subunit complexes need accessory or scaffolding proteins to promote productive associations. The requirement for these interactions may actually be to specifically create proper binding events or prevent incorrect or untimely events, or it may be necessary less specifically simply to hold subunits in a receptive state until they encounter their partner(s).

The classical example of a scaffolding function is that of assembly of bacteriophage (see also Introduction of Chapter 2). Assembly occurs by the orderly association of intermediates, which in turn interact to form the final product. Along the way, scaffolding proteins are present but then release, or are displaced, before the mature phage is finished. These scaffolding proteins are usually essential and required stoichiometrically. In some cases, such as the 44/62 protein in T4 DNA polymerase holoenzyme assembly, putative scaffolding proteins are required substoichiometrically (Kaboord and Benkovic, 1996). This "catalytic" role may imply that the scaffold is not depleted, but participates in rounds of binding reactions. One explanation for why scaffolding proteins are required in different stoichiometries could be the time in which the product is required, as a burst or gradual accumulation. Alternatively, the half life of the accessory protein may limit the number of events possible.

The bacterial flagellum is an example of coordinated assembly, in which there are a series of intermediates before the mature structure is finished. The

filament grows out of the hook. After the hook is complete, FliD attaches and promotes assembly of the filament by flagellin monomers (Homma and Iino, 1985; Ikeda et al., 1987). FliD remains associated with the distal end of the growing filament. A protein that performs a similar function in microtubule assembly has not been described, but would be difficult to detect. The stable association of FliD with the filament does not meet a strict definition of scaffolding. However, another step of flagellum assembly does provide an example of scaffolding. FlgD is required for the assembly of the hook from the first step. Using anti-FlgD antibodies and various hook mutants, Ohnishi et al. (1994) detect FlgD associated with intermediates of hook assembly. In fact it remains bound until the hook is complete, at which point it finally dissociates.

An interesting example of a eukaryotic scaffolding function occurs during histone assembly by chromatin assembly factor 1 (CAF1) (Kaufman et al., 1995). CAF1 binds to histones H3 and acetylated H4 and promotes their binding to replicating DNA, where they are joined by histones H2A and H2B (Kaufman et al., 1995). CAF1 is a complex of three proteins, at least two of which are necessary for activity. CAF1 will only act on newly synthesized histones H3 and H4 present in the cytoplasm and not on histones that are older and have entered the nucleus (Kaufman et al., 1995). This requirement may help ensure the correct ordering of the assembly pathway.

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CHAPTER 2

RBL2P, A YEAST PROTEIN THAT BINDS TO β-TUBULIN AND PARTICIPATES IN MICROTUBULE FUNCTION IN VIVO

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Rbl2p, a Yeast Protein That Binds to β-Tubulin and Participates in Microtubule Function In Vivo

Julie E. Archer, Leticia R. Vega, and Frank Solomon
Department of Biology
and Center for Cancer Research
Massachusetts Institute of Technology
Cambridge, Massachusetts 02139

Summary

Genetic configurations resulting in high ratios of β-tubulin to α-tubulin are toxic in S. cerevisiae, causing microtubule disassembly and cell death. We identified three non-tubulin yeast genes that, when overexpressed, rescue cells from excess β-tubulin. One, *RBL2*, rescues β-tubulin lethality as efficiently as does α-tubulin. Rbl2p binds to β-tubulin in vivo. Deficiencies or excesses of either Rbl2p or α-tubulin affect microtubule-dependent functions in a parallel fashion. Rbl2p has functional homology with murine cofactor A, a protein important for in vitro assays of β-tubulin folding. The results suggest that Rbl2p participates in microtubule morphogenesis but not in the assembled polymer.

Introduction

Cytoskeletal structures are constructed from a few basic polymers that are notable for the stringent and detailed conservation of their ultrastructure. Those polymers occur, however, in arrays with a wide range of geometries and functions. For example, microtubule organizations differ dramatically among cell types. Even in a single cell type, the microtubule arrays can vary in form and extent of assembly during development or upon passage through the cell cycle. An unresolved issue is an understanding of how cells specify the quantitative and qualitative variations in cytoskeletal assembly.

Regulation of microtubule assembly could occur at any of several places along the pathway. Divergent domains in the primary sequence of tubulin subunits could be crucial (Fuller et al., 1987). The amount of the individual subunits (Cleveland et al., 1981) and folding of the polypeptides to form assembly-competent dimers (Yaffe et al., 1992) may also be important. A variety of experiments demonstrate that activities that nucleate microtubule assembly (Oakley et al., 1990) and that stabilize microtubules by binding along their lengths (Caceres and Kosik, 1990; Dinsmore and Solomon, 1991) can contribute to microtubule function. The precise role and detailed mechanism of action of each of these factors are not yet well understood, nor is their contribution to the regulation of microtubule structure.

Genetic approaches provide valuable tools to identify important steps and essential components of morphogenetic pathways in vivo. A standard tool is the analysis of interacting mutations. An early and successful application of this sort of analysis is crucial to our understanding of phage assembly. Isolation of second-site revertants of mutant components identified interacting structural partners such as genes *1* and *5* in bacteriophage P22 (Jarvik and Botstein, 1975). For microtubules, second-site revertants of tubulin mutants identified γ-tubulin, a presumably ubiquitous and essential component of the microtubule-organizing center (Zheng et al., 1991), as well as proteins that may act along the length of microtubules (Pasqualone and Huffaker, 1994). This approach has been particularly useful in identifying genes that affect actin assembly in yeast (Adams and Botstein, 1989; Adams et al., 1989). These suppression events are likely to represent physical interactions.

An alternative genetic approach to a qualitative suppression analysis derives from quantitative considerations. The assembly of complex structures can require coordinated participation of multiple elements, some at intermediate steps and some in the final product. Again, phage studies demonstrate that successful assembly of complex structures may be sensitive to the relative levels of those components and require precise stoichiometries; an abnormal stoichiometry can lead to formation of aberrant and poisonous intermediates. For example, amber mutations in the T4 tail fiber gene *18* result in a lowered expression level of product, and mature phage progeny are not produced. Suppressors of this defect include amber alleles of interacting components (tail base plate genes) that result in lower, balanced levels of expression of the two components (Floor, 1970). This interaction is interpretable if one considers that the two gene products ordinarily interact and that a deficit in one of them leaves the other free to form otherwise forbidden interactions that lead to defects in assembly. Normal assembly, then, depends not on the absolute level of the gene products but rather a balance of components (Floor, 1970; Sternberg, 1976). The same sort of reasoning explains the requirement for balanced expression of histone proteins to produce normal chromosome segregation in the yeast Saccharomyces cerevisiae (Meeks-Wagner and Hartwell, 1986).

The details of tubulin expression in yeast present an opportunity to apply this analysis to microtubule assembly (Weinstein and Solomon, 1992). Genetic configurations that result in an increase in the ratio of β-tubulin to α-tubulin relative to wild-type cells are either toxic or lethal (Burke et al., 1989; Katz et al., 1990; Schatz et al., 1986). When β-tubulin is overproduced using an inducible galactose promoter on a 2μ (multicopy) plasmid, cells lose their microtubules within 1.5 hr, as assayed by immunofluorescence. Only 1% of the cells are viable after 4 hr, at which time the β-tubulin protein levels have only increased 2- to 4-fold. In contrast, the galactose-mediated induction of α-tubulin on a high copy plasmid does not affect microtubule assembly and becomes modestly toxic only after many hours and much higher levels of expression. However, restoration of the balance between α- and β-tubulin levels, by simultaneous overexpression, rescues both the

microtubule and cell lethality phenotypes associated with excess β-tubulin (Weinstein and Solomon, 1990).

It is not clear why β-tubulin in the absence of its normal partner, α-tubulin, affects microtubules and, presumably as a result, causes cell death. It may compete with αβ-tubulin heterodimers for growing ends of microtubules or for microtubule-associated proteins. It also may poison the nucleation site: shortly after the microtubules disappear in cells overexpressing β-tubulin, small foci of anti-β-tubulin but not anti-α-tubulin staining appear near the nucleus (Weinstein and Solomon, 1990); those dots colocalize with spindle pole body staining, using the anti-90 kDa spindle pole body component described by Rout and Kilmartin (1991) (M. Magendantz and F. S., unpublished data). By sequestering stabilizing factors or blocking nucleation sites, β-tubulin polypeptides may preclude native microtubule structure.

To identify proteins that interact with β-tubulin, we designed a screen to find genes whose products suppress the lethality associated with β-tubulin overexpression. Our rationale was that the overproduction of the target of β-tubulin, or more generally any β-tubulin-binding protein, would titrate the excess polypeptide and so allow polymer assembly and cell viability. We have identified three genes encoding proteins other than α-tubulin whose overexpression suppresses excess β-tubulin toxicity. One of them, here called *RBL2* (for rescues excess β-tubulin lethality), encodes a protein that rescues the excess β-tubulin phenotype as efficiently as does α-tubulin. Rbl2p is a β-tubulin-binding protein (the second identified, after α-tubulin). Its properties in vivo are similar to those of α-tubulin, and its levels affect microtubule functions. Rbl2p is a structural and functional homolog of cofactor A, a protein identified as necessary for an in vitro assay of tubulin folding (Gao et al., 1993, 1994). The results are consistent with an activity for Rbl2p in microtubule assembly at a step after folding but before dimerization.

Results

A Screen for Non-Tubulin Components of the Microtubule Assembly Pathway

To identify gene products that interact with β-tubulin, we screened for cDNAs that when overexpressed allowed cells to grow in the presence of excess β-tubulin. JAY47 is a diploid strain into which we integrated a third copy of the *TUB2* gene under control of the galactose promoter. This strain is indistinguishable from its wild-type parent in glucose, but in galactose it rapidly loses microtubule staining, arrests as large-budded cells, and dies with a half-life essentially identical to strains bearing pGAL-TUB2 on a 2μ plasmid (Weinstein and Solomon, 1990). We transformed a pGAL1-10-promoted yeast cDNA library (Liu et al., 1992) into JAY47 and selected colonies that were able to survive on plates with galactose as their sole carbon source (see Experimental Procedures). We isolated the plasmids from the suppressed JAY47 cells and sequenced the cDNA inserts.

The suppressing cDNAs encoded both of the yeast α-tubulins, Tub1p and Tub3p, and three other proteins. We have named the non-tubulin genes *RBL1*, *RBL2*, and *RBL3*. We evaluated their effectiveness as suppressors by comparing the number of colonies that arise on galactose (inducing) plates versus those on glucose (noninducing) plates (Table 1). For JAY47 cells containing a control plasmid, that ratio is 0.01%. By this assay, *RBL2* is as good a suppressor as either α-tubulin gene, *TUB1* or *TUB3* (Table 1); 70% of the *RBL2*-suppressed JAY47 cells can form colonies on galactose. The colonies are robust and uniform in size. Both *RBL1* and *RBL3* confer intermediate values of suppression (1% and 3%, respectively), and in both cases there is some variability in the size of the colonies. These characteristics of *RBL* suppression argue against a model in which there is a constant probability of death at each cell division, since that circumstance would predict a high percentage of colonies when growing on galactose, although small in size. An alternative explanation is that cells plated in galactose could face an early event at which the suppressed state can be established and thereafter maintained. In this sense, the effectiveness of the suppressors reported in Table 1 is a measure of their ability to establish suppression at early times.

The Sequences of the *RBL*s Suggest Different Functions

We cloned the genomic version of each of the *RBL* cDNAs and determined that each represented its full-length transcript. *RBL1* bears no homology to any sequence available in the database. The sequence of *RBL3* was entered in the database during the course of this study under the names *TIF3* (Altmann et al., 1993) and *STM1* (Coppolecchia et al., 1993). The gene product of *RBL3* is similar to human translation factor eIF4-B, although a direct assay of initiation activity is not yet available. Rbl2p is 32% identical and 61% similar at the level of predicted amino acid sequence to mouse cofactor A (Figure 1). Cofactor A is a necessary but not sufficient component required for α- and β-tubulin release from the chaperone t-complex polypeptide 1 (TCP-1) in a form competent for exchange into exogenous bovine tubulin dimer (Gao et al., 1993, 1994).

Table 1. *RBL1*, *RBL2*, and *RBL3* Suppress JAY47 Lethality

Plasmid	Number of Isolates	Colonies on Galactose / Colonies on Glucose
YCpGAL	NA	0.0001
TUB1 and *TUB3*	95	0.7
RBL1	1	0.01
RBL2	31	0.7
RBL3	1	0.03

Of 8.1 × 10^5 JAY47 cells containing the pGAL cDNA library plated on galactose medium, 950 survived to grow into colonies. Of these, 194 were plasmid dependent, and we have isolated 146 of these plasmids. The number of isolates column lists the representation of *TUB1* and *TUB3* and *RBL1*, *RBL2*, and *RBL3* among the plasmids recovered. The remaining 18 fail to suppress when retransformed into JAY47. Upon retransformation of the *TUB* and *RBL* plasmids, we determined their extent of suppression by plating cells to galactose (inducing) and glucose (noninducing) plates and comparing colony-forming units.

```
RBL2        MAPT...QLDIKVKALKRLTKEEGYYQQELKDQEAHVAKLKEDKSVDPYD 47
CofactorA   MADPHVRQIKIKTGVVRRLVKERVMYEKEAKQQEEKIEKMKAEDG.ENYA 49

            LKKQEEVLDDTKRLLPTLYEKIREFKEDLEQFL...KTYQGTEDVSDARS 94
            IKKQAEILQESRMMIPDCQRRLEAAYTDLQQILESEKDLEEAEEYKEARV 99

            AITSAQELLDSK 106
            VLDSVK..LEA. 108
```

Figure 1. Yeast Rbl2p and Murine Cofactor A Are 32% Identical

Comparison of predicted complete amino acid sequences of Rbl2p and cofactor A by the Genetics Computer Group program BESTFIT. Sequences are 32% identical and 61% similar across their entire lengths.

Effect of Overexpressing *RBL* Genes on Tubulin Levels

A potential mechanism for suppression of β-tubulin lethality is diminished accumulation of excess β-tubulin polypeptide due to effects at any point in its synthesis or on its stability. None of the *RBL*s appear to act in this manner. Protein samples harvested from galactose-induced JAY47 cells suppressed with each of the *RBL* plasmids contain an increased level of β-tubulin relative to noninduced cells, as judged by Western blot (Figure 2A). The result suggests that overproduced *RBL* gene products act by rendering the excess β-tubulin protein nontoxic to the cells. The α-tubulin levels remain constant for *RBL1* and *RBL2*, but increase modestly in cells with *RBL3* (Figure 2B). However, in wild-type cells, overexpressing *RBL3* does not increase steady-state levels of α-tubulin (data not shown), so we do not know whether it represents a direct effect on α-tubulin synthesis.

Our preliminary characterization suggests that the three *RBL* genes may act in quite different ways. We have chosen to focus on *RBL2*, which is as effective a suppressor as a previously known β-tubulin-interacting gene, *TUB1*.

Specificity of Genetic and Physical Interactions between Rbl2p and Tub2p

Excess Rbl2p does not act as a general suppressor of lethality resulting from the overexpression of other cytoskeletal genes. In particular, overexpression of Rbl2p does not rescue cells overexpressing either *ACT1* (encoding actin) or *TUB1* (α-tubulin; data not shown). This specificity and the similarity between the efficiency of suppression displayed by Rbl2p and α-tubulin suggest that Rbl2p may interact physically with the β-tubulin polypeptide.

The specificity of the genetic interaction is recapitulated by the results of immunoprecipitations from cells overexpressing Rbl2p and either α- or β-tubulin. We prepared total cell protein extracts from cells overexpressing both *RBL2* and either *TUB2* (JAY286) or *TUB1* (JAY381). We see Rbl2p expression increase by approximately 30-fold when induced behind a galactose promoter (data not shown). Each extract was incubated with antibodies against α- or β-tubulin or against Rbl2p, and the resulting precipitates were analyzed by immunoblots with antibodies against all three proteins (Figure 3). The antibodies against each of the tubulin polypeptides bring down the other chain with high efficiency. The results also demonstrate that approximately 5%–10% of total Rbl2p coimmunoprecipitates with β-tubulin when both are overexpressed in the same cells. In contrast, only 0.5% or less coprecipitates with anti-α-tubulin antibodies when those

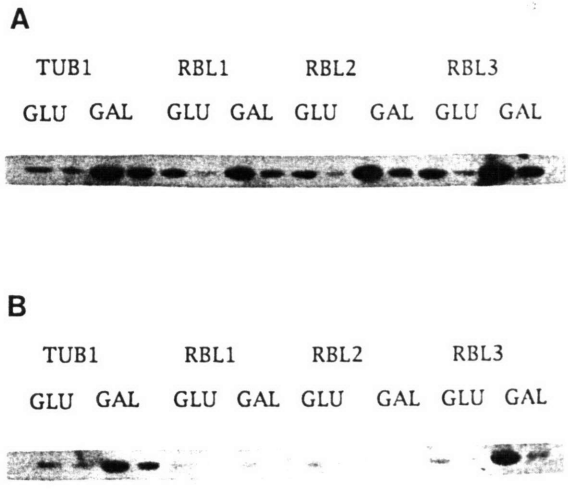

Figure 2. Levels of β- and α-Tubulin in Suppressed JAY47 Cells

JAY47 cells (diploids with an integrated pGAL-TUB2) containing pGAL-TUB1, pGAL-RBL1, pGAL-RBL2, or pGAL-RBL3 CEN plasmids were plated to galactose or glucose plates. We harvested colonies from galactose plates after 2.5 days or from glucose plates after 1.5 days and prepared total protein extracts. Samples representing 2× and 1× loads normalized to cell number were analyzed on 7.5% SDS–polyacrylamide gels. After transfer to nitrocellulose, β- (A) and α-tubulin (B) levels were assessed by Western blot using the polyclonal antibodies 206 and 345, respectively.

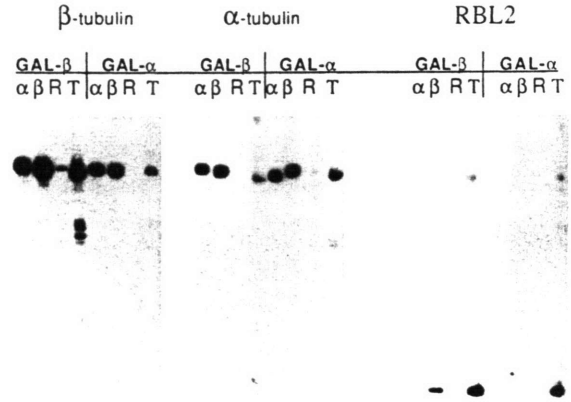

Figure 3. Rbl2p Coimmunoprecipitates with β-Tubulin

Cells containing inducible *RBL2* (CEN plasmid) and either inducible *TUB2* (JAY286) or *TUB1* (JAY381) on 2μ plasmids were grown in raffinose and then shifted to 2% galactose for 8 hr. Total protein extracts and relevant immunoprecipitates were analyzed by immunoblotting after resolution on three parallel 12% polyacrylamide gels. β-Tubulin, α-tubulin, and RBL2 indicate the antibodies used for blotting; GAL-β and GAL-α are the strains co-overexpressing either Rbl2p and β-tubulin or Rbl2p and α-tubulin, respectively. Lanes contain protein precipitated with anti-α-tubulin (α), anti-β-tubulin (β), or anti-Rbl2p (R). Lane T contains total cell protein, representing one-fourth the material in the immunoprecipitates.

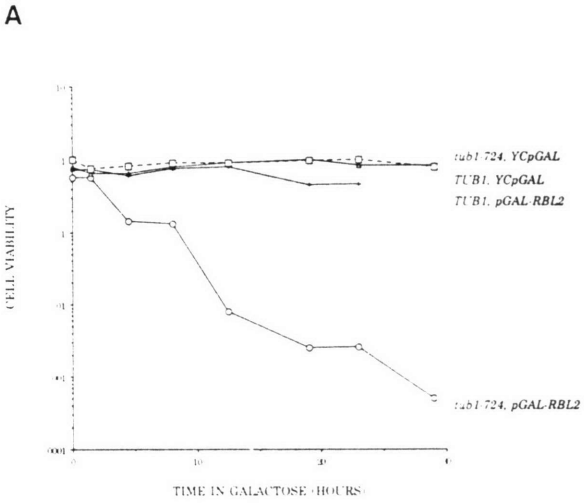

Figure 4. Synthetic Interaction of *RBL2* Overexpression with *tub1-724*

(A) Haploid cells contain two plasmids each: either *TUB1* or *tub1-724* on a CEN plasmid as their only source of α tubulin and either inducible *RBL2* or YCpGAL (control) CEN plasmid. These strains were grown overnight in selective raffinose media at 30°C. At t = 0 hr, galactose was added to 2%. Cell viability equals the number of colonies arising on glucose plates divided by cell number counted in a light microscope. (B and C) At t = 5 hr in galactose, *tub1-724* cells containing either control (B) or *pGAL-RBL2* (C) plasmids were fixed and processed for immunofluorescence with anti-β-tubulin antibody 206. In control cells, there are a variety of tubulin staining patterns. In cells overexpressing Rbl2p, large-budded cells contain little or no localized staining.

two proteins are overexpressed. The same specific association is apparent when anti-Rbl2p antibodies are used. Much more β-tubulin than α-tubulin is present in anti-Rbl2p precipitates from the respective overproducing strains. We precipitate the same specific complex, although with lower efficiency, in strains overexpressing only Rbl2p but none of the tubulin genes. In strains not overexpressing Rbl2p, we fail to detect coimmunoprecipitation, probably because endogenous levels of Rbl2p are so low.

We can detect no colocalization of Rbl2p with assembled microtubule structures in cells. In both wild-type cells and in strains overproducing Rbl2p, antibodies against the protein do not give a discretely localized signal by immunofluorescence microscopy (data not shown). Instead, anti-Rbl2p antibodies do stain Rbl2p-overexpressing cells very brightly, suggesting that the antibodies can recognize cellular Rbl2p after fixation. We conclude that the failure to detect a discrete signal probably reflects a diffuse localization of the protein. Therefore, the apparent association of Rbl2p and β-tubulin is likely to occur with unassembled tubulin chains rather than assembled microtubules.

Microtubule Defects Are Sensitive to the Level of Rbl2p

The overproduction of Rbl2p in wild-type cells leads to a modest loss of viability. After 10 hr of induction, about 80% of the cells are not viable, but the effect levels off at that point. However, overexpression of Rbl2p in some backgrounds with compromised microtubules greatly enhances this lethality. For example, we previously described a panel of α-tubulin mutants (Schatz et al., 1988), several of which are conditional lethals that arrest with no microtubules at low temperature and are supersensitive to the microtubule-depolymerizing drug benomyl at permissive temperatures. Overexpression of Rbl2p at permissive temperature in one such mutant strain, *tub1-724*, causes rapid and nearly complete cell death (Figure 4A). One other *tub1* allele, *tub1-728*, shows a similar loss of viability when Rbl2p is overexpressed, while several *tub1* alleles show no such interaction (Table 2). This lethal interaction also causes a dramatic loss of microtubules. Figure 4 also shows immunofluorescence micrographs of *tub1-724* in the absence (Figure 4B) or presence (Figure 4C) of excess Rbl2p for 5 hr.

The phenotype of *RBL2* overexpression is recapitulated by *RBL2* null alleles. *RBL2* is not essential for mitotic growth, but it has a synthetic lethal phenotype in combination with four *tub1* alleles, but not with four others (Table 2). Two of those four alleles that do interact genetically with the *ΔRBL2* null, *tub1-724* and *tub1-728*, are the ones that enhance the lethality of excess Rbl2p.

Table 2. Synthetic Lethality of *RBL2* Overexpression and Null Strains

Allele	*RBL2* Overexpression	Δ*RBL2*
tub1-724 and *tub-728*	–	–
tub1-738 and *tub-759*	+	–
tub1-704, tub-714, tub-744, and *tub-750*	+	+
tub1-727, tub-730, tub-733, tub 741, tub-746, and *tub-758*	+	ND

Ability of mutants to grow at permissive temperatures upon induction of pGAL-RBL2 or in *RBL2* nulls. ND, not determined.

That both excess and absence of the *RBL2* gene product affect viability and probably microtubule assembly in these different genetic backgrounds suggests that it acts as a structural rather than catalytic element in microtubule assembly. In addition, the allele specificity of the interaction with mutant *tub1* alleles indicates that the combinatorial defect represents a more proximal functional interaction than simply two defects in unrelated processes.

The Stoichiometry of Rbl2p to Tubulin Is Critical

As noted above, any genetic configuration that results in an excess of β-tubulin over α-tubulin is toxic. Changes in the level of Rbl2p expression affect the phenotypes associated with changes in α-to-β tubulin ratios. Overexpression of Rbl2p suppresses excess β-tubulin lethality; similarly, when overexpression of β-tubulin is induced in strains bearing a deletion in *RBL2*, the cells lose viability with more rapid kinetics than strains wild type for *RBL2* (Figure 5A).

In the converse direction, extra Rbl2p also rescues the phenotypes produced by creating a modest deficit in α-tubulin. Strains bearing a deletion of the quantitatively minor α-tubulin gene, *TUB3*, are viable but benomyl supersensitive (Schatz et al., 1986). The enhanced sensitivity to this microtubule-depolymerizing drug is suppressed by a modest increase in Rbl2p (Figure 5B). This result fulfills our expectation that excess β-tubulin is lethal because of its stoichiometry relative to α-tubulin rather than its absolute level. Therefore, Rbl2p levels appear to compensate for the defects associated with either too much β-tubulin or too little α-tubulin.

Rbl2p Levels Affect Cellular Sensitivity to the Microtubule-Depolymerizing Drug Benomyl

In a sense, the phenotypes of excess β-tubulin mimic those of benomyl; both lead to loss of microtubules, cell cycle arrest as large-budded cells, and death. This parallel is supported by the consequences that changes in the level of either Rbl2p or α-tubulin have on either poison. Extra Rbl2p or α-tubulin (Schatz et al., 1986) produced by galactose induction confers resistance to benomyl (Figure 6A). Conversely, the absence of Rbl2p renders cells more sensitive to the drug (Figure 6B). A modest decrease in α-tubulin levels produced by disruption of the *TUB3* gene also produced supersensitivity to benomyl (Schatz et al., 1986).

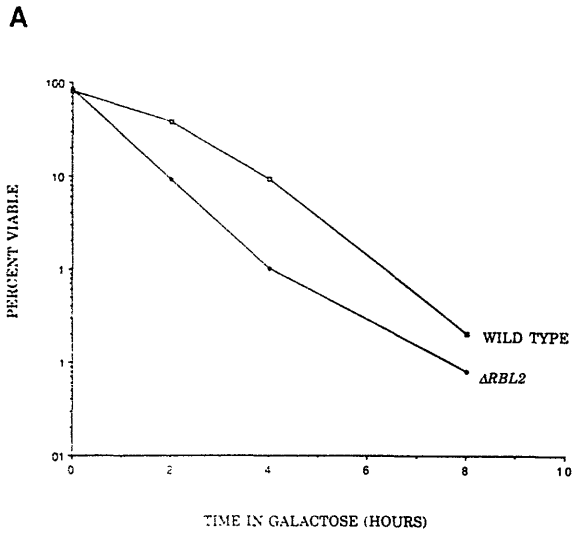

Figure 5. Phenotypic Consequences of Altered Stoichiometry between Rbl2p and Tubulin

(A) Effects of Rbl2p levels on sensitivity to β-tubulin overexpression. Haploid cells with an integrated copy of inducible *pGAL-TUB2* (derivatives of JAY47) either wild type for *RBL2* or bearing a null allele were grown and analyzed for viability as described in Figure 4.

(B) Effects of excess Rbl2p on the benomyl sensitivity of cells with a deficit in α-tubulin. Δ*TUB3* haploids (FSY21) containing either control (minus) or genomic *RBL2* (plus) on a CEN plasmid were serially diluted on plates containing 10 μg/ml benomyl. Dilutions were by halves, beginning at 10^8 cells per milliliter in the first two columns and at 10^7 cells per milliliter in the second two columns.

The phenotypes of *RBL2* overexpression do not appear to be manifestations of multidrug resistance because they do not include resistance to several other drugs, such as cycloheximide and ethidium bromide (data not shown). This phenotype is further remarkable because it is uncommon; enhanced resistance to this drug suggests a degree of specificity for microtubule function not inherent in enhanced sensitivity.

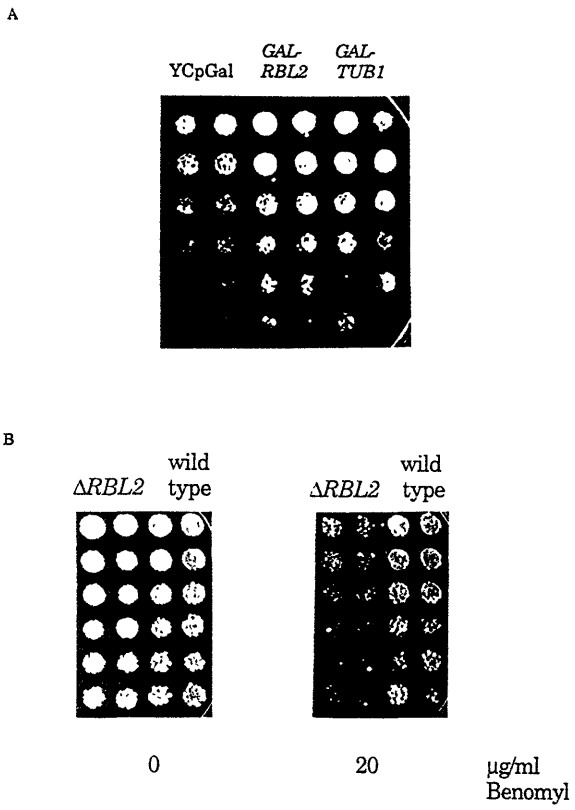

Figure 6. Levels of Rbl2p Affect Growth on Benomyl, a Microtubule-Depolymerizing Drug

(A) Diploids cells containing control (YCpGAL), pGAL-RBL2, or pGAL-TUB1 CEN plasmids were serially diluted on selective galactose plates with 20 μg/ml benomyl. Dilutions were by halves, beginning at 10^7 cells per milliliter.

(B) *ΔRBL2* haploid cells containing control or genomic *RBL2* CEN plasmids were serially diluted as in (A) on 0 and 20 μg/ml benomyl plates.

Rbl2p Is Important for Meiosis

Although *RBL2* is not essential for mitosis, it is necessary for cells to complete sporulation and meiosis successfully. We made diploids that were heterozygous or homozygous for *ΔRBL2*. Both grew normally. However, the *ΔRBL2* homozygotes sporulate abnormally; they produce asci with only slightly reduced efficiency, but substantially fewer of those asci contain four spores (Table 3). Those spores vary significantly in size and are arranged in a disorderly fashion. In contrast, the heterozygotes sporulate comparably to wild-type diploids. We examined this defect with an assay for haploid spores, using the difference in color between *ADE2* and *ade2* colonies. We sporulated strains that were heterozygous at the *ADE2* locus. Colonies from either *ADE2* haploids or unsporulated *ADE2/ade2* diploids are white; cells bearing only the *ade2* allele are red. We recovered no red colonies from the homozygous *RBL2* nulls. The same strain containing *RBL2* on a low copy plasmid produced red colonies at an efficiency indistinguishable from wild type (35%–40%).

A Functional Homology between Rbl2p and Murine Cofactor A

The predicted protein sequence of Rbl2p is approximately 30% identical to mouse cofactor A across their entire lengths (see Figure 1). Cofactor A is thought to participate in chaperonin-mediated folding of β-tubulin in vitro (see Discussion). To determine the relationship between Rbl2p and cofactor A, we expressed mouse cofactor A in yeast. Like excess Rbl2p, overexpression of this sequence in yeast cells confers substantial resistance to excess β-tubulin lethality (Figure 7A); the efficiency of suppression is approximately 5%, compared with 70% for the yeast protein. In addition, murine cofactor A suppresses the benomyl supersensitivity associated with deletions of *RBL2* (Figure 7B). These results suggest that cofactor A performs overlapping functions with Rbl2p.

Discussion

We identified Rbl2p in a screen for proteins that, when overexpressed, protect cells from the deleterious effects of β-tubulin overexpression. We envisioned at least two possible sorts of suppressing elements that would answer this screen. One might be a protein with which excess β-tubulin interacts to cause microtubule disassembly, which might include associated proteins or nucleating elements, but also might include tubulin itself. For example, γ-tubulin acts as a nucleator of microtubules and interacts genetically with β-tubulin (Oakley and Oakley, 1989). However, overexpression of the presumptive yeast γ-tubulin *TUB4* (L. Marschall and T. Stearns, personal communication) does not rescue β-tubulin lethality (data not shown). Another suppressor might be a protein with which undimerized β-tubulin interacts normally, as part of the morphogenetic pathway. The interaction between actin mono-

Table 3. *ΔRBL2/ΔRBL2* Cells Have a Defect in Sporulation and Meiosis

Strain	Plasmid	Percent Asci	Spores per Ascus				
			Four	Three	Two	One	Zero
Wild type	None	71	69	9	9	2	11
ΔRBL2/ΔRBL2	*RBL2*	56	47	13	12	4	24
ΔRBL2/ΔRBL2	Control	49	18	24	18	12	28

After 4 days in sporulation media (1% potassium acetate [pH 7]) at room temperature, each population was scored for the number of spores within asci. We designated cells as asci based on their rounded shape and thickened cell coat and counted the number of spores contained within. The category of zero spores per ascus met the criteria for an ascus but looked either empty or murky inside. The strains are either wild type (FSY185) or *ΔRBL2/ΔRBL2* homozygotes containing either a *RBL2* genomic or control CEN plasmid. We counted >300 cells for each.

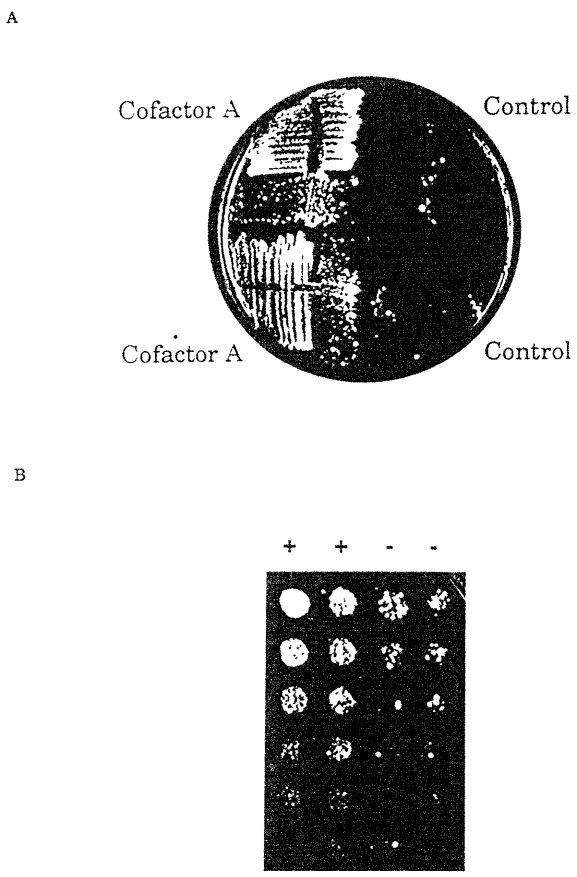

Figure 7. Overexpression of Murine Testes Cofactor A Has Phenotypes Reminiscent of Rbl2p in Yeast

(A) JAY47 cells containing pGAL cofactor A (CEN plasmid) are suppressed relative to those containing control CEN plasmid on galactose plates.

(B) *ΔRBL2* haploids transformed with pGAL cofactor A (plus) or control (minus) CEN plasmid serially diluted on galactose benomyl (25 μg/ml) plates by halves, beginning at 10^7 cells per milliliter.

mers and profilin may serve as a precedent (Magdolen et al., 1993). The experiments described above suggest that Rbl2p may be in the second class of potential suppressors. The suppression and immunoprecipitation data argue for an intimate interaction between Rbl2p and β-tubulin. The binding of Rbl2p to β-tubulin probably explains the suppression of excess β-tubulin by Rbl2p. The aggregate genetic evidence suggests that Rbl2p acts along the microtubule assembly pathway. But the fact that Rbl2p cannot be detected in the end product of this pathway, assembled microtubules, implies that it participates in some intermediate along the pathway.

Scaffolding Proteins and Surveillance Functions

Assembly of bacteriophage proceeds through intermediates that can contain scaffolding proteins, elements that participate in the maturation of the particle, but which are not incorporated into the final structure. For instance, the prohead of P22 grows around a core of gp8 molecules that are later replaced by DNA while the coat proteins expand to arrange themselves as the head (King et al., 1973). Some properties of Rbl2p suggest that it may act as a scaffolding protein in microtubule assembly. For example, the stoichiometry of a scaffolding protein would be predicted to be important for ensuring proper assembly. Although Rbl2p is not essential for mitosis, aberrant levels of the protein are deleterious when microtubule integrity is impaired: genetic backgrounds bearing mutant α-tubulins or in the presence of benomyl. However, there is no evidence that scaffolding proteins are required in the formation of microtubule polymer from an αβ heterodimer. No intermediates involving transiently associated proteins have been identified, and, at least in vitro, tubulin heterodimers can self-assemble. Perhaps, then, Rbl2p acts between the synthesis of the β-tubulin polypeptide and its incorporation into active dimer. The physical interaction with β-tubulin and the specificity of the suppressor activity of excess Rbl2p for β-tubulin are consistent with Rbl2p binding to the β-tubulin monomer. Alternatively, Rbl2p could act transiently during remodeling of microtubules among different organizational states. In any of these sites of action, Rbl2p might mediate progress through the assembly process, like a phage scaffolding protein.

Another possibility is that the screen that identified Rbl2p represents its function in cells: Rbl2p might act as a β-tubulin ligand to suppress deleterious interactions under aberrant conditions in which β-tubulin monomers occur. Such a surveillance function could quite adequately be executed by α-tubulin itself. However, significant excesses of α-tubulin are deleterious and do not persist (Katz et al., 1990; Weinstein and Solomon, 1990). In addition, a role for a β-tubulin-binding protein may become more important under special circumstances, for example, when the α-tubulin gene product is compromised in mutant *tub1* strains.

Functional Similarities between Rbl2p and α-Tubulin

We find striking similarities between the properties of Rbl2p and α-tubulin in vivo (Figure 8). The ability of Rbl2p, like α-tubulin, to bind β-tubulin is itself strong evidence of function. An excess of either protein suppresses β-tubulin lethality and confers resistance to the microtubule-depolymerizing drug benomyl. Deficiencies of either enhance sensitivity to benomyl (Schatz et al., 1986). Extra Rbl2p can actually compensate for a quantitative defect in α-tubulin: cells lacking the minor α-tubulin gene are supersensitive to benomyl, but are rescued by genomic *RBL2* on a low copy plasmid. Finally, the phenotypes of α-tubulin mutants are strongly affected by levels of Rbl2p. In sum, Rbl2p is a β-tubulin-binding protein in vivo, and that binding is detectable both physically and functionally.

The benomyl resistance could be explained if that drug acts by promoting dissociation of the αβ dimer to release free β-tubulin that poisons the microtubules in the cell. However, two other suppressors of excess β-tubulin, *RBL1* and *RBL3*, fail to confer benomyl resistance. Another possibility is that the binding of benomyl to tubulin creates toxic drug–dimer complexes analogous to those that may

	LEVEL OF Rbl2p OR α–TUBULIN EXPRESSION		
	DEFICIENCY	NORMAL COMPLEMENT	EXCESS
Condition:			
NORMAL	<*	=	<*
tub1	<^	=	<^
β-OVEREXPRESSION	<^	=	>*
Δtub3	n.d.	=	>*
BENOMYL	<*	=	>*

Figure 8. Alterations in the Level of Rbl2p or α-Tubulin Have Similar Effects on Cell Growth Under a Variety of Conditions

The lesser-than symbol denotes inhibition of growth by a change in the level of Rbl2p relative to the normal complement; the greater-than symbol represents better growth. The asterisk denotes those circumstances in which changes in the level of α-tubulin have the same effects as Rbl2p. The carat indicates those experiments not done (n.d.) with α-tubulin.

be the active species in inhibition of microtubule assembly by colchicine (Skoufias and Wilson, 1992). These toxic complexes might be sequestered by the activity of excess Rbl2p or α-tubulin.

The meiotic requirement for Rbl2p may be due to a greater reliance on events that are in fact common to both meiosis and mitosis. Cells undergoing mitosis even in the absence of Rbl2p may be safely above a threshold for an essential component, for example, an assembly-competent tubulin dimer or a Rbl2p functional homolog, whereas meiosis may change that threshold so that the contribution of Rbl2p becomes required. Cells may be more sensitive to free β-tubulin during meiosis. Alternatively, Rbl2p may perform an essential meiosis-specific function. We note that although cofactor A mRNA is present in many mouse tissues, it is most abundant in testes (Gao et al., 1993). Although this observation originally was explained as reflecting a role for cofactor A in constructing sperm flagellae, instead it may reflect an increased dependence on mouse cofactor A in meiosis itself.

A Comparison with Cofactor A

Cofactor A is a polypeptide that, together with a fraction called cofactor B, is necessary for the release of α- and β-tubulin from the chaperone TCP-1 in a form competent for exchange into exogenous bovine tubulin heterodimer (Gao et al., 1993, 1994). TCP-1 alone can bind both unfolded actin and γ-tubulin polypeptides and release them in a form that migrates normally on a native gel. TCP-1 and TCP-1-like proteins may play an important role in actin and tubulin function in vivo. Complexes containing TCP-1 and actin or tubulin can be isolated from animal cells (Sternlicht et al., 1993). Strains bearing mutant alleles of TCP-1 homologs can exhibit microtubule and actin phenotypes in yeast (Chen et al., 1994; Vinh and Drubin, 1994). In the in vitro assay, proper tubulin folding is assayed not by the measure of folded monomer, but instead by incorporation of the monomer into tubulin dimers. It requires the addition of native tubulin dimers. The presence of cofactor A results in the appearance of β-tubulin dissociated from TCP-1; in contrast, cofactor A does not have the same effect on α-tubulin dissociation from TCP-1. The specificity for β-tubulin strongly implies that cofactor A is not involved in common protein folding pathways. If TCP-1 acts as does GroEL (repetitive binding of unfolded forms until they do fold properly in solution [Weissman et al., 1994]), Rbl2p could act after the release step, to capture and stabilize folded β-tubulin. That model is consistent with our results, with the failure of cofactor A to promote α-tubulin release from the same TCP-1, and with the failure of others to detect any direct interaction between cofactor A and TCP-1 (Gao et al., 1994).

In summary, a screen for overexpressed wild-type genes that restore balance to the components of microtubule assembly identified a stoichiometrically acting component that behaves like a scaffolding element. Changes in the level of Rbl2p exacerbate circumstances that compromise microtubule assembly, suggesting that it may act at a crucial and regulatory step in microtubule morphogenesis.

Experimental Procedures

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 (Sherman et al., 1986; Solomon et al., 1992). We used a yeast cDNA (CEN) library from pool 10A provided by H. Liu (Liu et al., 1992). pJA10 was constructed with the PvuII–EagI (pGAL-RBL2) fragment of pA5 into the PvuII–EagI backbone of YEp13 (*LEU2*, 2μ). pJA34 was constructed by isolating mouse cofactor A from a FVB mouse adult testes cDNA library (provided by D. Page) by PCR and cloning the fragment into the SalI–NotI backbone of the pGAL-CEN library plasmid (Liu et al., 1992).

Screen for Suppressors of β-Tubulin Lethality

We transformed 10A into JAY47 and obtained approximately 6.8 × 10^4 original transformants. We grew the transformants in selective glucose media to saturation (expansion of $>10^6$-fold). We tested 950 galactose survivors for dependence on the plasmid by selecting for loss of the *URA3* plasmid on 5-FOA and then checking for loss of suppression. After isolation of the library plasmid, we identified those that contained either *TUB1* or *TUB3* by a combination of restriction digests, colony hybridization, and DNA sequencing. The isolated plasmids were retransformed into JAY47 and checked for their ability to confer survival on galactose plates.

DNA Sequencing

DNA sequencing on both the cDNA inserts and genomic versions was performed using modified T7 DNA polymerase Sequenase with the dideoxy chain termination method (U. S. Biochemical Corporation). The genomic clones were isolated either from 2μ yeast genomic libraries RB378 and 380 (Carlson and Botstein, 1982) or from a CEN yeast genomic library prepared by C. Thompson and R. Young (Massachusetts Institute of Technology [MIT]). The genomic clones and cDNAs match exactly, indicating that both contain the entire open reading frames.

Immune Techniques

Antibody Production and Purification

A glutathione S-transferase–Rbl2p fusion protein was overexpressed using pGEX-5X (Pharmacia) in Escherichia coli, purified and injected into three rabbits, and boosted at 2, 4, and 6 weeks. Anti-Rbl2p antibodies (248, 249, and 250) were affinity purified against the fusion protein. Rabbit antisera against β-tubulin (206) and α-tubulin (345) are described elsewhere (Weinstein and Solomon, 1990).

Immunofluorescence

We used standard procedures (Solomon et al., 1992). Secondary antibody was fluorescein isothiocyanate–conjugated goat anti-rabbit IgG (Cappel). For Rbl2p staining, we also tried extracting fixed cells after attaching to the slides with 0.5% NP-40 in PM2G followed by using

a 0.1% BSA blocking step (M. Magendantz and F. S., unpublished data), by using methanol/acetone fixation (Rout and Kilmartin, 1991), and by varying the time in formaldehyde (10 min to 2 hr) or in first antibody (1–16 hr). DAPI (4',6-diamidino-2-phenylindole dihydrochloride; Boehringer Mannheim) was used to visualize DNA.

Immunoblots

We used standard procedures (Solomon et al., 1992). After gel electrophoresis and transfer to nitrocellulose membranes, blots were blocked blots 3% BSA, PBSA, sodium azide for 30–120 min. Primary antibodies were incubated for >12 hr at 1/3500 (206 or 345) or at 1/100 (250) and then washed five to seven times (5 min each) in 2% hemoglobin, 0.1% SDS, 0.05% NP-40. Bound antibody was detected by [^{125}I]protein A (New England Nuclear).

Immunoprecipitations

Antibodies were affixed to Affigel-10 beads (Bio-Rad). Yeast strains JAY286 and 381 are FSY185 transformants with two 2μ plasmids: pGAL-RBL2 (pJA10) and either pGAL-TUB2 (pBW54) or pGAL-TUB1 (pQX3). Total protein was harvested by French Press in PME (0.1 M PIPES, 2 mM EGTA, 1 mM magnesium chloride [pH 6.9]) plus protease inhibitors (Solomon et al., 1992) and added to antibody beads for a 1 hr incubation with rotation at 4°C. We washed the beads eight times with PME plus protease inhibitors.

Genetic Analyses

Construction of ΔRBL2

We replaced SnaBI–XhoI of pA21A (genomic *RBL2* plasmid), which completely removes the *RBL2* open reading frame. with SspI–SalI of pNKY51 (Alani et al., 1987), which contains *URA3* flanked by *hisG* repeats for efficient loopout. We used an SspI–MunI disrupting fragment to create a heterozygous knockout in FSY185, confirmed by Southern blot analysis. We sporulated and dissected the heterozygote to produce haploid *ΔRBL2* cells, confirmed by Southern and Western blot analysis.

Synthetic Lethality

For *RBL2* overexpression, FSY185 (wild-type diploid) or haploid strains containing *tub1* alleles were transformed with pA5. Transformants were grown overnight in selective raffinose media, and then galactose was added. Viability was assessed at various times by comparing cell number by hemocytometer count to colony-forming units on glucose plates. Alternatively, differences could also be assessed by plating strains to galactose plates and comparing cell number and size.

The *ΔRBL2* JAY422 strain (*ΔRBL2* haploid) was crossed to haploid *tub1* mutants. The diploids were sporulated and dissected. Synthetic interactions were judged by two criteria: percent of dead spores and marker analysis (inability to recover *tub1* allele plus *ΔRBL2* products).

Assays for Meiosis

JAY472 and 474 are transformants of a heterozygous *ADE2/ade2*, homozygous *ΔRBL2/ΔRBL2* strain (JAY428) with A21A (*RBL2* genomic CEN plasmid) or with a control CEN plasmid. JAY472, JAY474, and FSY185 (wild-type) cells were grown to midlog, washed with water, and shifted to 1% potassium acetate (pH 7). Sporulating cells were incubated rotating at room temperature. By visual inspection at 4 days, we scored the presence of asci containing zero to four spores. The criteria for an ascus were rounded shape and thickened cell wall. JAY474 has spore sacs that usually contain fewer than four spores. For quantitation of appearance of *ade2* cells, we allowed strains to sporulate, digested the cell walls with Zymolyase-100, and plated. After 3 days we counted the number of red colonies and the number of total colonies.

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GenBank Accession Number

The accession number for the sequence reported in this paper is U30184.

CHAPTER 3

ANALYSIS OF THE PHYSICAL ASSOCIATION OF RBL2P AND β-TUBULIN

INTRODUCTION

Levels of Rbl2p are important for microtubule functions, but Rbl2p acts on β-tubulin that is not assembled (see Chapter 2; Archer et al., 1995). Therefore, Rbl2p must function with a different mechanism than a conventional MAP. Microtubules assemble reversibly *in vitro*, beginning with either cell extracts or pure tubulin subunits. These reactions only partially represent the problem of microtubule assembly *in vivo*. For example, spatial and temporal constraints and mandates based on internal geometries are relieved outside the context of the intracellular environment. Furthermore, tubulin must achieve the proper ternary and quaternary structure, steps not important in the *in vitro* assay.

α- and ß-tubulin cannot be easily produced in recombinant form, because they form insoluble aggregates (Zabala and Cowan, 1992; Gao et al., 1993). The identification of the TCP-1 family as a GroEL-like cytoplasmic chaperonin suggested an explanation to this problem (Frydman et al., 1992). Yeast strains with altered forms of TCP-1 genes exhibit cytoskeleton defects. TCP-1 forms a large hetero-oligomeric complex responsible for folding a subset of cellular proteins, including tubulins (Lewis et al., 1992). Whereas γ-tubulin achieves a folded state in the presence of TCP-1 alone, α- and ß-tubulin

require the presence of additional cofactors (Gao et al., 1993; Campo et al., 1994). Cofactor A (a Rbl2p homolog) and cofactor B allow the release of α- and ß-tubulin from the chaperonin complex in forms that are competent for exchange into exogenous heterodimer (but see below). Cofactor A alone promotes the recovery of ß-tubulin from the chaperonin, as assayed by native gel, but this state of β-tubulin can not exchange into dimer (Gao et al., 1994) . Cofactor B might influence the state of either new ß-tubulin, dimerized tubulin, or exchange per se. Recently, the Cowan lab has found that exchange of β-tubulin into dimer requires three cofactors (C, D, and E) in the presence or absence of cofactor A, and these cofactors are not sufficient for the exchange of α-tubulin into dimer (Tian et al., 1996) . Although Cowan and co-workers originally proposed that cofactor A is a co-chaperonin, our work on *RBL2* demonstrates that Rbl2p acts on ß-tubulin rather than TCP-1 (see Chapter 2; Archer, et al., 1995) . Recent work on mouse cofactor A is also consistent with the model that it interacts with ß-tubulin as a distinct step in an assembly pathway, independent of the chaperonin-mediated folding (Tian et al., 1996) .

Here we address issues concerning the form of ß-tubulin that is a substrate for Rbl2p binding. Previously, we demonstrated that Rbl2p co-immunoprecipitates with β-tubulin but not α-tubulin (Chapter 2), a result that suggests that Rbl2p associates selectively with ß-tubulin. However, α-

and ß-tubulin themselves always co-immunoprecipitate. Because both α-tubulin and Rbl2p are present in the anti-β-tubulin immunoprecipitates, we could not rule out the possibility that α-tubulin was involved in the Rbl2p-β-tubulin interaction. The absence of Rbl2p from anti-α-tubulin immunoprecipitates could be the result of an artefact, such as steric hindance by the antibody. We now used tagged Rbl2p as the probe for interactions. We find that Rbl2p preferentially binds to monomeric ß-tubulin in vivo or in vitro. Furthermore, it can compete with α-tubulin for binding to ß-tubulin because it associates with ß-tubulin from heterodimer, apparently displacing α-tubulin. With this result and others, we show that Rbl2p most likely interacts with a nearly native form of β-tubulin.

MATERIALS AND METHODS

Plasmids, Strains, and Media

pQE-60/RBL2 was used to produce recominant Rbl2(His6)p in *E.coli*. It was constructed with the use of PCR to add NcoI and BglII site right before the start codon and after the stop codon, respectively. The PCR primers were as follows: 5'TAGGACACCATGGCACCCACACAATTG3' and 5'AATCTGAGATCTTTTAGATCGAGTAATTC3'. The PCR product was

cloned into the NcoI and BglII sites of Qiagen vector pQE-60. The same design was used to create pQE-60/mouse cofactor A.

pGRH allows inducible expression of Rbl2(His6)p in *Saccharomyces cerevisiae.* pQE-60/RBL2 was digested with HindIII and blunted, then digested with MfeI. This fragment was cloned into pA5 (*URA3*, *CEN*, GAL1-10 promoter, see Archer et al., 1995) that was digested with NotI and blunted, then digested with MfeI.

pQE-60/DHFR was used to produce recombinant dihydroxyfolate reductase (Magendantz et al., 1995) .

All yeast strains are deriviatives of FSY185 (Weinstein and Solomon, 1990) . JAY570 is JAY47 plus pGRH. JAY614 is FSY185 plus pGRH. We used standard methods and media (Sherman et al., 1986; Solomon et al., 1992) . Isolation of recombinant proteins from E. coli was as in Magendantz et al. (1995).

Purification of His6-tagged Proteins

The Ni-NTA nickel slurry and columns materials are from Qiagen. We used protocols that are slight modifications of Magendantz et al. (1995) and the Qiagen handbook.

In vivo association experiments: We grew JAY570 or 614 overnight in selective raffinose media, then induced with galactose for 10-16 hours. We harvested protein by French Press (Solomon et al., 1992), using approximately 4 X 10^9 cells per experiment. We used a buffer (PME plus protease inhibitors) volume equal to the volume of the pellet. We applied 1 ml of protein extract to 500µl Ni-NTA slurry that was pre-incubated with buffer VI (40 mM imidazole, 300 mM NaCl, 50 mM sodium phosphate buffer, pH8.0). After a 1-2 hour rotating incubation at 4°C, we washed three times with 10 ml buffer I or buffer VI (20 or 40 mM imidazole). Finally, we either added the slurry to a Quiagen Ni-NTA column and eluted specifically with 1 ml 200 mM imidazole or did not load a column but added 1 ml buffer and boiled off bound proteins.

In vitro association experiments: We harvested protein from FSY185 (wild type), FSY272 (overexpresses β-tubulin) or FSY274 (overexpresses α-tubulin). After breaking the cells with a French Press, we immediately added 300-500µl recombinant Rbl2(His6)p, then proceeded as above.

Binding of denatured proteins: We harvested protein from FSY185. We treated 1 ml of sample with 6M guanidine hydrochloride for 5-15 minutes on

ice. We diluted the sample (or untreated control) 100 fold into PME plus protease inhibitors plus 500µl recombinant Rbl2(His6)p, then proceeded as above.

Dissociation experiments: We prepared β-tubulin-Rbl2p complex from JAY570 protein extract. After washing the nickel slurry as above, we resuspended the samples in PME plus protease inhibitors (100 fold dilution). At various times, we spun down aliquots of the samples, added buffer I and boiled to remove bound proteins.

GTP cross-linking

GTP binding and cross-linking was modified from V. Praitis (docotoral dissertation, 1995). Rbl2p-β-tubulin complex was isolated from JAY570 or JAY614 protein extract as above. After elution, we combined 15 µl eluate with 5 µl of Hot GTP mix (2X MES (80mM MES, pH 6.4, 0.8mM EGTA, 0.4mM $MgCl_2$), 160 µM ATP, 0.3 µM ^{32}P α-GTP(Dupont-NEN), 4 µM GTP). After incubation on ice for 30 minutes, we cross-linked the bound GTP with 2 X 999.9 mJoules at a distance of 3 cm in a Stratagene UV Stratalinker 2400. We immediately added loading buffer and boiled the samples.

Immunoblots and silver staining

Acrylamide gels were all 12% acrylamide. We used antibodies 206 (anti-β-tubulin) at 1/3500, 345 (anti-α-tubulin) at 1/1750, and 250 (anti-Rbl2p) at 1/100. Silver staining was standard (Magendantz et al., 1995).

RESULTS

Rbl2p associates with monomeric ß-tubulin *in vivo*

We constructed a six-histidine tagged Rbl2p which we either harvested from *E.coli* for use *in vitro* or expressed in yeast allowing *in vivo* associations to occur. The His6 tag allows Rbl2(His6)p to bind to nickel-containing beads with high affinity because of the electrostatic interactions formed in the coordination sphere. We performed experiments to identify proteins that, by virtue of their association with Rbl2p, would also be retained on the nickel beads.

This modified form of Rbl2p (Rbl2(His6)p) appears to act indistinguishably from untagged Rbl2p, as judged by several assays of phenotypes associated with RBL2 (see chapter 2). The overexpression of Rbl2(His6)p rescues the lethality of excess ß-tubulin. A plasmid containing inducible *RBL2(His6)*, suppresses the overproduction of *TUB2* to an efficiency of 49%, compared with

70% by pA5 (untagged *RBL2*) and 0.01% by YCpGal (control plasmid). These numbers represent viable cells on inducing versus non-inducing plates. Similarly, Rbl2(His6)p overexpression confers resistance to benomyl (data not shown).

From cells co-overexpressing ß-tubulin and Rbl2(His6)p, we observe the binding of ß-tubulin and Rbl2(His6)p to nickel beads (Figure 3-1). This result is consistent with the presence of a ß-tubulin Rbl2p complex and is, in effect, the reciprocal experiment to the co-immunoprecipitation of Rbl2p with anti-β-tubulin antibodies (see chapter 2). However, in this case, essentially no α-tubulin is present. This absence strongly supports the idea that Rbl2p binds a monomeric, undimerized form of ß-tubulin.

Rbl2p preferentially associates with ß-tubulin *in vitro*

The association of Rbl2p and ß-tubulin can be recapitulated *in vitro*. We harvested protein extracts from strains overproducing only ß-tubulin or α-tubulin and incubated them with recombinant Rbl2(His6)p and nickel beads. Again, ß-tubulin binds to Rbl2(His6)p and is retained on the beads (Figure 3-2).

Figure 3-1. ß-tubulin binds to Rbl2p in vivo.

We harvested protein from strain JAY570 overexpressing ß-tubulin and Rbl2(His6)p and incubated it with nickel slurry (see Materials and Methods). After elution from the nickel, we analyzed the samples by Western blot. Duplicate lanes were probed with antibodies against ß-tubulin, α-tubulin and Rbl2p. "Protein" is the original protein preparation, "eluate" is the population that was present after imidizole elution.

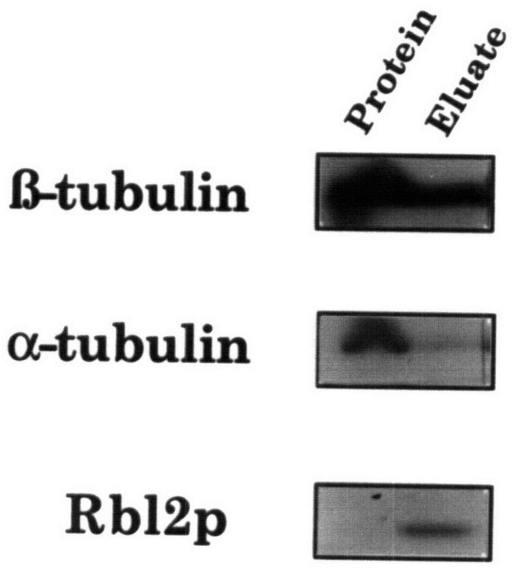
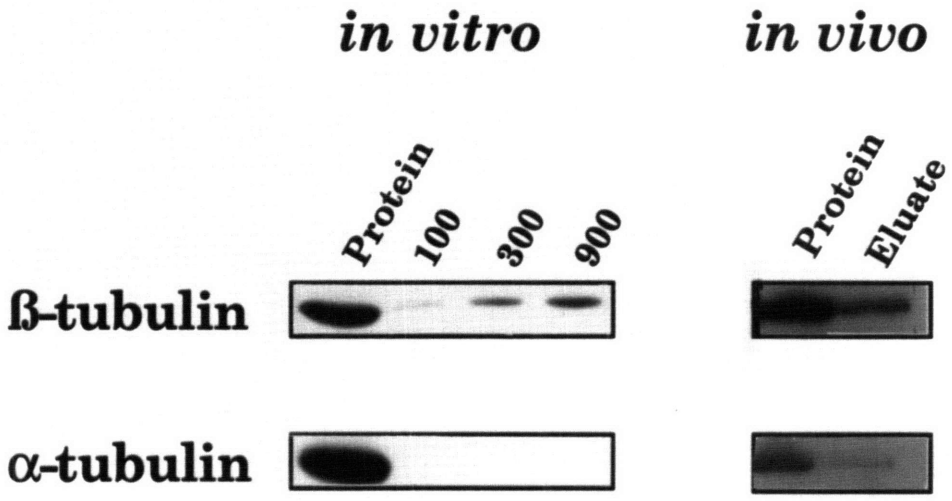

Protein
Eluate
ß-tubulin
α-tubulin
Rbl2p

Figure 3-2. ß-tubulin binds to Rbl2p in vitro.

We harvested protein from strains overexpressing ß-tubulin or α-tubulin and incubated them with recombinant Rbl2(His6)p and nickel slurry (see Materials and Methods). After elution from the nickel, we analyzed the samples by Western blot. Duplicate lanes were probed with antibodies against ß-tubulin, α-tubulin and Rbl2p.

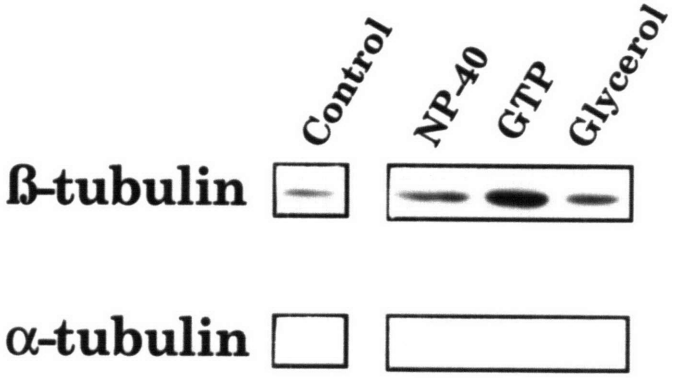

in vitro
in vivo
Protein
100
300
900
Protein
Eluate
ß-tubulin
α-tubulin

Rbl2p may recognize a nearly native form of ß-tubulin

The previous experiments demonstrate that ß-tubulin is a substrate for Rbl2p when β-tubulin has been overproduced. The overexpression of ß-tubulin creates a sizeable pool of undimerized ß-tubulin of unknown conformation. In order to assess Rbl2p's ability to associate with endogenous ß-tubulin, we performed *in vivo* and *in vitro* association experiments using strains with wild type tubulin levels. We induced Rbl2(His6)p in an otherwise wild type strain, or we added recombinant Rbl2(His6)p to wild type extract. Again, we see ß-tubulin retained on the nickel beads (Figure 3-3). In fact, approximately 1-10% of the cellular ß-tubulin is associated with Rbl2p. Increasing the amount of recombinant Rbl2(His6)p leads to increasing amounts of β-tubulin retained (Figure 3-3 "in vitro"). From native gel analysis, others have shown that essentially all of the pool of tubulin in a cell runs with the mobility of a dimer. Therefore, the ability of Rbl2p to associate with ß-tubulin in these experiments suggests that it is interacting with ß-tubulin that has previously been dimerized and hence fully folded. Although there may be conformational alterations prior to or subsequent to Rbl2p binding, the complex does form from ß-tubulin which has been competent to interact with α-tubulin. Moreover, Rbl2p is effectively competing with α-tubulin for binding to β-

Figure 3-3. Rbl2p binds to ß-tubulin from wild type cells.

We harvested protein from wild type strains in which tubulin levels were not manipulated. We added recombinant Rbl2(His6)p to wild type FSY185 protein extract ("in vitro") or overexpressed Rbl2(His6)p before protein harvest in JAY614 ("in vivo"). In the in vitro experiment, we added various amounts of recombinant protein (arbitrary units of 100, 300, and 900). We incubated the samples with nickel slurry and eluted, before Western blot analysis (see Materials and Methods). Duplicate lanes were probed with antibodies against ß- and α-tubulin.


Control
NP-40
GTP
Glycerol
ß-tubulin
α-tubulin

tubulin. The absence of α-tubulin in the complex suggests the possibility that these complexes with β-tubulin are mutually exclusive.

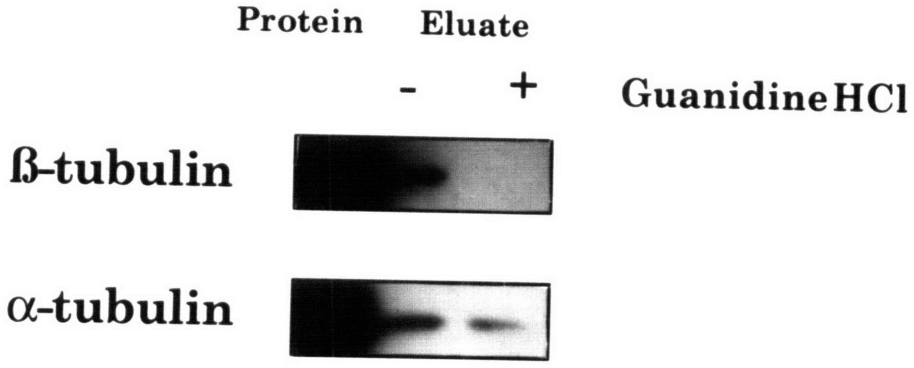
Conversely, denatured ß-tubulin is a poor substrate for Rbl2p. Conventional chaperone binding and folding assays often make use of substrates that are denatured by treatment with 6M guanidine hydrochloride. We treated wild type yeast protein with guanidine hydrochloride and asked what was the fate of the denatured tubulin when diluted into recombinant Rbl2(His6)p. Relative to control samples which have been diluted but not denatured, ß-tubulin binding is substantially reduced (Figure 3-4). α-tubulin, on the other hands, associates to a similar extent in the presence or absence of guanidine hydrochloride. Moreover, an unidentified band that routinely cross-reacts with our anti-α-tubulin antibodies, binds to a greater extent after denaturation (data not shown). So, β-tubulin's ability to specifically associate with Rbl2p is dependent on its conformation and is abolished upon denaturation.

Rbl2p and ß-tubulin disassociate with slow kinetics

We analyzed the stability of the Rbl2p-β-tubulin complex. After isolating the complex on nickel beads, we diluted it into buffer and monitored loss from the

Figure 3-4. ß-tubulin is not a good substrate for Rbl2p after treatment with guanidine hydrochloride.

We performed binding experiments with wild type extract that had been pre-treated "+" (or not "-") with 6M guanidine HCl before dilution into buffer containing recombininant Rbl2(His6)p. We analyzed samples by Western blot with antibodies against ß- and α-tubulin.

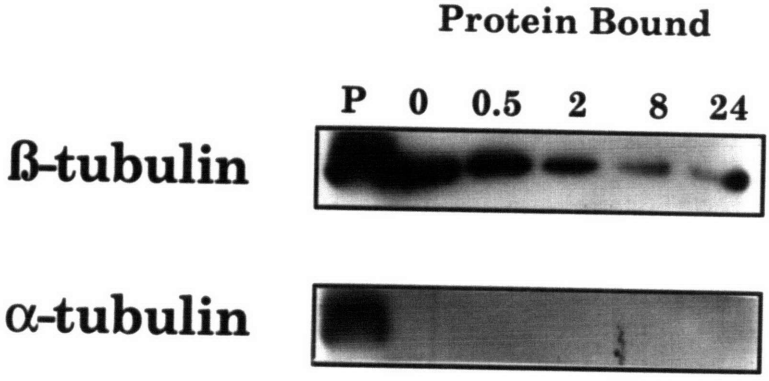

Protein
Eluate
-
+
Guanidine HCl
ß-tubulin
α-tubulin

beads. The complex is quite stable; ß-tubulin dissociates at only a modest rate (Figure 3-5). We estimate the half-life of the complex to be approximately 1 hour or 3.6 X 10^3 seconds. k_{off} is approximately $0.693/t_{1/2}$, so for Rblp2-β-tubulin would equal 2 X 10^{-4} sec^{-1}. If we make the assumption that k_{on} is diffusion rate limited and use the very conservative value of 10^6 M^{-1} sec^{-1} for k_{on}, then Rbl2p and β-tubulin have a K_D of approximately 2 X 10^{-10} M^{-1}. In comparison, α and ß-tubulin have a K_D of 2 X10^{-7} as assayed by sedimentation coefficient equilibrium techniques. While this estimate of K_D for α-β tubulin hererodimer is problematic because of the time involved for the technique, this comparison does suggest that the Rbl2p-β-tubulin complex is fairly stable and perhaps roughly comparable to that of α- and β-tubulin.

Because there are no other characterized β-tubulin binding proteins (except perhaps Cin1p (Tian et al., 1996)), we currently have nothing to which to compare this rate. It will be interesting to measure the half life of complex produced from overexpressed β-tubulin versus that of complex created from existing dimer. This comparison may suggest the similarity (or not) or overexpressed β-tubulin conformation to that of nearly native β-tubulin.

Figure 3-5. Kinetics of dissociation of ß-tubulin and Rbl2p.

We harvested protein from cells overproducing ß-tubulin and Rbl2p and isolated the complex on a nickel slurry. At time "0" we diluted the nickel-bound complex into buffer. At various times thereafter (0, 0.5, 2, 8. and 24 hours), we removed aliquots of the nickel beads. We analyzed the protein remaining associated with the nickel beads by Western blot with antibodies against ß- and α-tubulin.


Protein Bound
P
0
0.5
2
8
24
ß-tubulin
α-tubulin

GTP affects the association of Rbl2p and ß-tubulin

We addressed the effect that agents known to influence tubulin and microtubule assembly have on tubulin-Rbl2p association. We incubated recombinant Rbl2(His6)p with wild type yeast extract in the presence of 0.1% Nonidet P-40, 2 M glycerol or 1 mM GTP. In a different assay, detergents such as NP-40 promote dimer disassociation, while glycerol and GTP help maintain association (B. Weinstein, doctoral dissertation; V. Praitis, doctoral dissertation). Under our conditions, NP-40 and glycerol did not affect the binding of ß-tubulin to Rbl2p, but GTP increased the extent of association (Figure 3-6).

Based on cross-linking experiments, ß-tubulin is said to contain an exchangeable GTP binding site (Nath and Himes, 1986) . We were interested to determine if the ß-tubulin that is associated with Rbl2p is competent for exchange of GTP. We isolated Rbl2p-β-tubulin complexes, as above, incubated them with radioactive GTP, and examined the position of signal after cross-linking. We do observe GTP crosslinking to a protein that runs at the same mobility as ß-tubulin (Figure 3-7). We also see other cross-linked bands although Rbl2p, the most abundant protein in the sample, does not

Figure 3-6. GTP affects the association of ß-tubulin with Rbl2p.

We harvested wild type protein extract and added recombinant Rbl2(His6)p and nickel slurry in the presence of nothing ("control") or 0.1% NP-40, 1 mM GTP, or 2M glycerol. We analyzed the protein that bound by Western blot analysis with antibodies against ß- and α-tubulin.

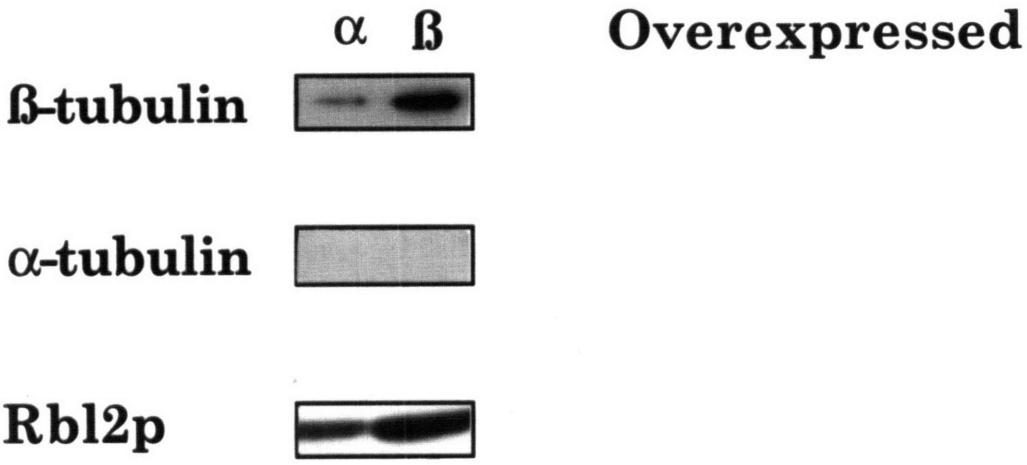
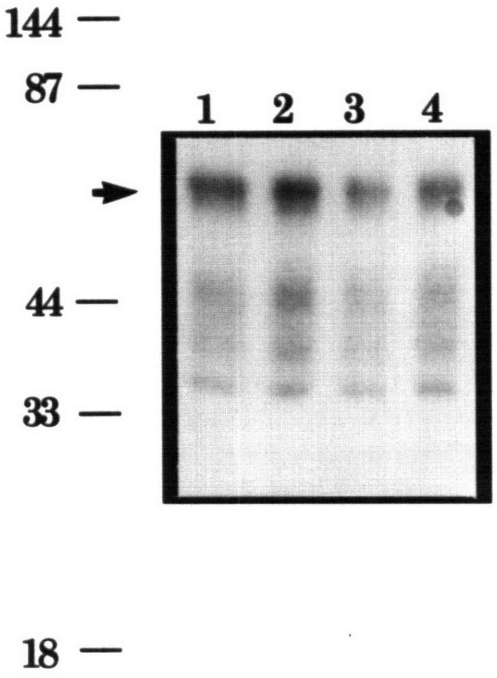

α ß
Overexpressed
ß-tubulin
α-tubulin
Rbl2p

Figure 3-7. GTP cross-linking to ß-tubulin associated with Rbl2p.

We isolated complex as in Figures 3-1 (JAY570) and 3-3 (JAY614). We cross-linked radiolabelled GTP to the samples (see Materials and Methods), and examined the location of signal after SDS-PAGE. Lanes 1 and 2 are two JAY570 samples, lanes 3 and 4 are two JAY614 samples. The arrow indicates the position of β-tubulin as judged by Western analysis.


144
87
1
2
3
4
44
33
18

acquire radiolabel. Because more ß-tubulin binds when it is overexpressed, we tested if these samples would also cross-link to more radioactive GTP. We compared the signals that co-migrate with ß-tubulin in samples from single Rbl2p overexpressors versus samples double Rbl2p and ß-tubulin overexpressors. Note that background bands should be constant in level and GTP incorporation. We fail to see a reproducible increase in radioactive signal in the ß-tubulin overproducers (Figure 3-7), although we clearly see an increase in ß-tubulin associated with the nickel beads (data not shown, but see 3-1 versus 3-3). There are several possible explanations. It could be that GTP exchange is only occuring on the dimer ß-tubulin; α-tubulin "contamination" is constant between the samples, and therefore so is signal. Alternatively, GTP exchange could be limited by time. Perhaps exchange is slow and has not proceeded to completion. It could also be that free ß-tubulin is different from dimerized ß-tubulin; for instance perhaps Rbl2p bound ß-tubulin is competent for GDP-GTP exchange but not for GTP acquisition. This model predicts that the level of GDP-β-tubulin is comparable whether or not ß-tubulin is over-produced.

Rbl2p associates with a subset of cellular proteins

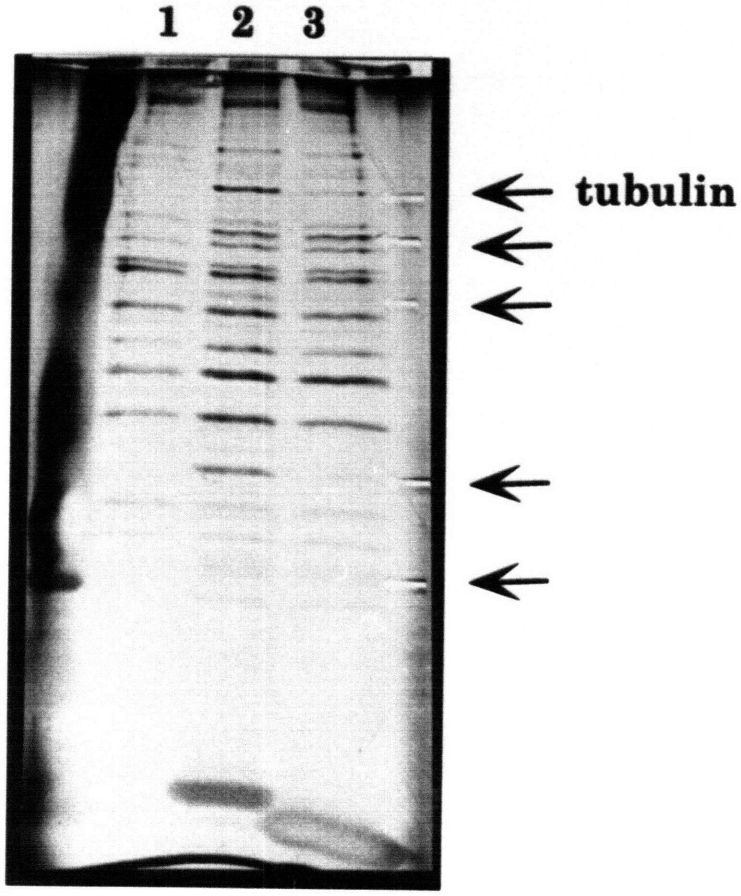
In order to address the uniqueness of the β-tubulin-Rbl2p interaction, we examined the number of proteins that associate with nickel beads in the

presence of Rbl2(His6)p. We incubated yeast extract with His6-tagged dihydroxyfolate reductase (DHFR, Figure 3-8 lane1), mouse cofactor A (lane 2), or Rbl2p (lane 3) and nickel beads. After silver staining analysis, we find a discrete subset of cellular proteins are retained in the presence of cofactor A or Rbl2p, but not DHFR. We found no examples of bands present only in cofactor A or Rbl2p, but not the other. In most cases, the bands appear more intense in the cofactor A sample than the Rbl2p sample, although the samples contain approximately equal amounts of recombinant Rbl2p or cofactor A as judged by silver or Ponceau S stain (data not shown). We do not yet know why this is the case.

DISCUSSION

In these experiments, we have primarily addressed the form of ß-tubulin that Rbl2p interacts with. Our results are consistent with the interaction involving a nearly native form of monomeric ß-tubulin. The ability of Rbl2p to bind to ß-tubulin which has recently been in dimerized form suggests it recognizes a folded conformation. Further evidence is the decreased association of ß-tubulin with Rbl2p when α-tubulin is over-expressed. Again,

Figure 3-8. Several proteins bind to both Rbl2(His6)p and cofactor A-His6. We harvested wild type protein extract and added recombinant DHFR-His6 (lane 1), cofactor A-His6 (lane 2), or Rbl2(His6)p (lane 3). We incubated the samples with nickel slurry and eluted the bound proteins. We examined the eluted samples by silver stain after SDS-PAGE (12% acrylamide gel). The arrows represent bands present in the cofactor A and Rbl2p lane with much greater intensity than in the DHFR lane. "Tubulin" indicates the position of β-tubulin as judged by Western analysis.


1
2
3
tubulin

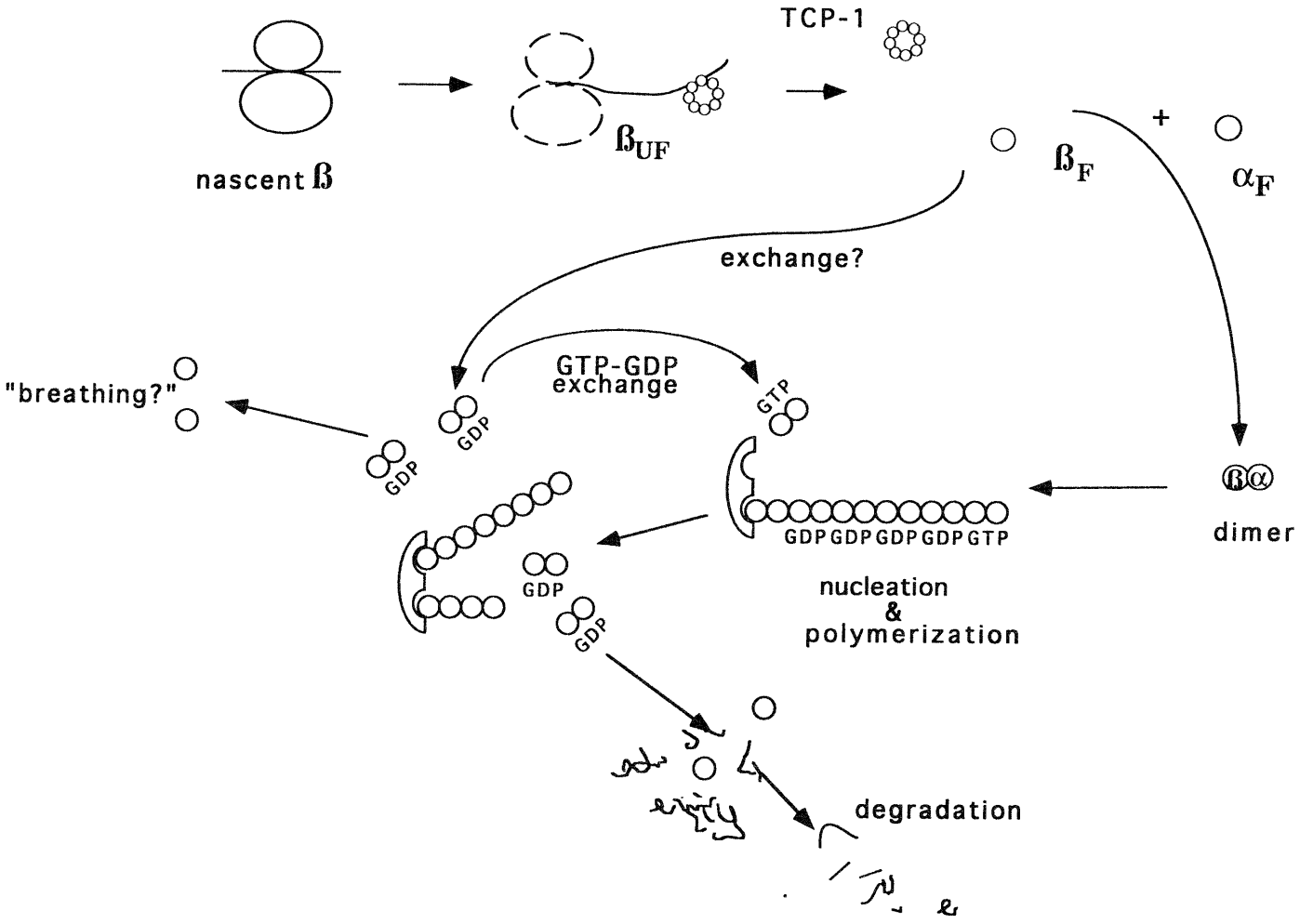
this result is most consistent with the two interacters recognizing the same form of ß-tubulin, or at least two easily convertible ones. It may be possible for chaperone substrates to acquire significant structure while associated with the chaperone. In folding reactions with TCP1 complex and α-tubulin, α-tubulin can obtain a conformation that allows GTP binding and that confers protection against proteolytic digestion (Tian et al., 1995) . In the case of TCP1 and β-tubulin, however, β-tubulin remains quite susceptible to proteolytic digestion until it is associated with cofactor A or cofactor D (Tian et al., 1996) .

Our results suggest that the binding of Rbl2p and α-tubulin to ß-tubulin are mutually exclusive events. Unfortunately, direct competition experiments are impossible in this system, because α- and ß-tubulin co-purify. It is difficult to work with components of obligate dimers, because any separation technique may well produce monomers that are abnormal or aggregated. However, it might be informative to define binding sites using polypeptide fragments and compare minimal required domains of the three proteins in question. We can compare the affinities of Rbl2p versus α-tubulin and see that they are quite different. This difference is expected because ß-tubulin is normally associated with α-tubulin and because the over-expression of Rbl2p is only modestly toxic.

Given that ß-tubulin is a GTP binding, as assayed by cross-linking experiments, it is intriguing that the presence of GTP affects the association of Rbl2p with ß-tubulin. One model for the effect of GTP would be that the nucleotide alters the association of α- and ß-tubulin within the dimer, so that ß-tubulin is a better substrate for Rbl2p. One result inconsistent with this model is that GTP appears to inhibit disassociation of α- and ß-tubulin in a different assay (B. Weinstein, doctoral dissertation; V. Praitis, doctoral dissertation). However, the ability of Rbl2p to catch freed tubulin subunits may alter the outcome of putative GTP-assisted dimer dissociation events. A second model, or perhaps a refinement of the first model, is that GTP plays a role in the maintainance of ß-tubulin conformation. Therefore, while it might stabilize existing dimer, it could also assist in holding monomeric ß-tubulin in a form that Rbl2p recognizes and hence increase the yield of Rbl2p-β-tubulin complex.

Rbl2p may function in a microtubule assembly pathway, assisting in a ß-tubulin specific step such as monomer sequestration, conformation maintainance, GTP acquisition or dimerization with α-tubulin. However, it is also possible that in addition or instead, Rbl2p acts at a dimerized or post-dimerized stage. During dimer dissociation or microtubule disassembly

Figure 3-9. Model of potential sites of Rbl2p-β-tubulin interaction (see text for details).


TCP-1
nascent ß
ßUF
ßF
+
αF
exchange?
"breathing?"
GTP-GDP
exchange
GDP
GDP
GTP
GDPGDPGDPGDPGTP
ßα
dimer
GDP
GDP
nucleation
&
polymerization
degradation

Rbl2p might stabilize or sequester ß-tubulin which is temporarily unpartnered. The dissociation from α-tubulin could be accidental or requisite for tubulin activity. Perhaps Rbl2p assists in the exchange of GDP for GTP, for instance. Finally, Rbl2p could escort monomeric ß-tubulin on a course to degradation. To monitor any preference for ß-tubulin at various points in its assembly pathway (Figure 3-9), it will be interesting to explore the timing of *in vivo* association. These experiments will use constitutive expression of Rbl2(His6)p under its own promoter as a probe while ß-tubulin can be manipulated using distinguishable forms (wild type and "tailless"). Both forms bind to Rbl2(His6)p (data not shown). We can identify a large pool of freshly synthesized β-tubulin with this inducible system because it can be immunologically distinguished from the constitutive pool that will consist of "tailless" β-tubulin. With only the freshly synthesized β-tubulin as the experimental sample, we can monitor appearance of polypeptide in the total protein and compare when it is able to associate with α-tubulin (as assayed by anti-a-tubulin immunoprecipitation) versus with Rbl2(His6)p (as assayed by nickel-binding).

Finally, the ability to isolate nearly native ß-tubulin may make it possible to obtain crystallographic information about this protein. High resolution structural information about any tubulin is not presently available and would

be of value to the field.

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CHAPTER 4

RKS1, A GENE THAT INTERACTS WITH *RBL2* AND AFFECTS MICROTUBULES *IN VIVO*

INTRODUCTION

The apparent "essentiality" of cofactors in biochemical assays of tubulin folding/dimerization in vitro is in contrast to the *in vivo* result that *CIN1* and *RBL2* are not essential genes. One possibility is that spatially and temporally the environment for a freshly synthesized tubulin chain is simply different *in vivo*. For example, perhaps the physiological production of α- and β-tubulin occurs in a coordinated fashion so that monomers always immediately have partners, thus obviating the need for Cin1p and Rbl2p. Perhaps the requirements of the assay, namely the exchange into heterodimer, are not reflective of normal tubulin production. Another possibility is that the difference in requirements might reflect differences of translating polypeptide versus denatured full length protein (Frydman and Hartl, 1996) . Yet another possibility is that there are proteins whose functions overlap with Cin1p and/or Rbl2p. This last model predicts that it might be possible to identify such a redundant or cooperative activity through a synthetic lethal approach.

We have screened for mutants that are lethal in combination with a deletion of *RBL2*. We previously demonstrated that a subset of existing *TUB1* alleles exhibit synthetic lethality with Δ*rbl2* (Chapter 2). This screen identified

seven alleles of α-tubulin, plus another mutation we named *rks1-1* (*RBL2* Knockout Synthetic lethal). This mutant is temperature sensitive and loses microtubules at the non-permissive temperature. We have identified two suppressors of *rks1-1*. A deletion of one, here called *SUP1*, is also temperature sensitive with a concomitant loss of microtubules. Therefore, we have identified at least one new locus that appears to play a role in microtubule stability.

MATERIALS AND METHODS

Strains, plasmids and media

We used standard methods and media (Sherman et al., 1986, Solomon et al., 1992) . JAY414 or JAY553 are haploids strains containing complete disruptions of the chromosomal *RBL2* open reading frame and the centromeric plasmid pA21A with *RBL2* and *URA3* (Archer et al., 1995). pJA33 is a plasmid containing *RBL2* and *HIS3*. We constructed pJA33 by ligating the Sal1-Not1 insert of pA21A into Sal1-Not1 of pRS313 (Sikorski and Hieter, 1989) . We cloned SUP1 (pJ2 and pJ4) and SUP2 (pJ1, pJ3 and pJ5) as genomic inserts from the pCT3 library (prepared by C. Thompson and R. Young. MIT). pJA38 is a plasmid containing *SUP1* and *HIS3*. We constructed pJA38 by digesting pJ2 with SalI and PvuII and cloning the 2.6

kb fragment into a SalI-SmaI cut pRS313 backbone. pJA44 is a plasmid containing SUP1 under the control of the GAL1-10 promoter. We constructed pJA44 by using PCR to create SalI and NotI sites immediately before the start codon and after the stop codon, respectively. We used primers: 5'CCGGTCGACGGCCAATGTCAATAAGCGAAACACCTC3' and 5'CCGGCGGCCGCTCATTATACAGGCGGAGGTGGGGC3'. We digested the PCR product with SalI and NotI and ligated it into SalI-NotI digested pA5 backbone (Archer et al., 1995). pSET1 and pSET3 are plasmids containing HA epitope-tagged SUP1. We constructed pSET1 and pSET3 by inserting a NotI site immediately before the stop codon in pJA38. We then cloned a triple HA-epitope (M.Tyers, G. Tokiwa and B. Futcher) into the NotI site. pSET1 received two tandem inserts, and thus contains six HA epitopes. pSET3 has three HA epitopes. To assess rescue by *TUB1*, we transformed the strains with pRB539.

Mutagenesis

We mutagenized JAY414 or JAY553 with ethylmethane sulfonate (EMS) according to standard protocols (Guthrie and Fink, 1991) . After mutagenesis, we resuspended the cells in rich media at approximately 2 X 10^6 cell/ml. We plated an aliquot immediately for viability determination and grew the rest overnight at 30°C. The next day, we diluted the saturated culture 1/50 in rich media and allowed it to reach saturation before beginning

the screen. After isolation of synthetic lethal strains, we backcrossed them to a wild type haploid (FSY183 or FSY184).

Immune techniques

We used standard procedures for immuoblots and immunofluorescence (Solomon et al., 1992; Archer et al., 1995). We used anti-β-tubulin antibody #206 at a dilution of 1/2000 to assess the state of microtubules *in rks1-1* and Δ*sup1* cells. We used monoclonal antibody 12CA5 (BABCO) at a dilution of 1/1000.

DNA Sequencing

We obtained DNA sequence from the original SUP1 and SUP2 clones using universal primers T3 and T7. Sequencing was performed using modified T7 DNA polymerase Sequenase with the dideoxy chain termination method (U.S. Biochemical Corporation).

Disruption of *SUP1*

We created a plasmid for a complete disruption of *SUP1* by inserting the 5' and 3' non-coding regions around a *LEU2* marker. We isolated the 3' non-coding region immediately following the stop codon by PCR with the primers: 5'CCGTCTAGACATGATAAAGAAAAATTAGATTTCAAGCTATGTACC3'

and 5'CCGAAGCTTGGAGTGCGTTTATGTAGCTGCCGTC3'. The product has XbaI and HindIII ends that we used to clone into pJH-L2 that was digested with XbaI and HindIII (this step named pJA42). Next we isolated SUP1 5' non-coding region immediately before the start codon by digesting pJ2 with MscI and SacI. We cloned this fragment into pJA42 that was digested with SmaI and SacI (now named pJA43). For the integrating disruption, we digested pJA43 with PvuII and purified the 3 kb fragment. For Southerns to check for proper integration, we digested genomic DNA with EcoRI and probed with ^{32}P labelled 3' non-coding *SUP1* PCR fragment (see above). Note that the *LEU2* replacement of *SUP1* introduces a new EcoRI site so the new band is smaller than wild type.

RESULTS

Identification of genes synthetic lethal with Δ*rbl2*

We screened for genes that are synthetic lethal with Δ*rbl2*. We began with strains JAY414 or JAY553, which are haploids containing Δ*rbl2* covered with pA21A (*RBL2*, *CEN3* and *URA3*). In two rounds of this screen, we mutagenized this background with ethylmethane sulfonate resulting in a viability of 58 or 66%. In total, we plated 25,000 cells on rich medium and replica plated to 5-fluoroorotic acid (5-FOA) to select for the ability to lose

pA21A. We restreaked individual candidates to confirm their status as 5-FOA$^-$. Next, we transformed candidates with pJA33, which provides *RBL2* (in a *HIS3* vector) and so should relieve the requirement for pA21A. Eight strains were 5-FOA$^-$ in the absence and 5-FOA$^+$ in the presence of pJA33 (Figure 4-1). All eight strains are recessive for their synthetic lethality and conditional phenotypes (described below).

Of these eight strains, one is temperature sensitive, six are benomyl supersensitive, and one is both benomyl supersensitive and temperature sensitive (Figure 4-2). All seven benomyl supersensitive strains are relieved of their need for *RBL2* by an extra copy of *TUB1* (data not shown). This rescue suggests that the affected locus in these strains is *TUB1* or *TUB3*, although formally it is possible that *TUB1* is an extragenic suppressor of the true mutant locus. Crosses with a marked TUB1 strain also yield results that are consistent with *tub1* allelism (P. Alvarez and F. Solomon, unpublished results).

The temperature sensitive strain is not rescued by TUB1 for either its temperature sensitivity or its synthetic lethality (Figure 4-3). We have therefore provisionally named the affected locus *rks1*, for *RBL2* Knockout Synthetic lethal.

Figure 4-1. Eight strains require *RBL2* to live.

We streaked eight haploids strains on plates containing 5-FOA. The strains are Δrbl2 plus *RBL2* on a *URA3* plasmid (pA21A), so if they require *RBL2* they cannot lose the plasmid and grow on 5-FOA plates. We transformed the cells with *HIS3* vector backbone (pRS313) or containing *RBL2* (pJA33).

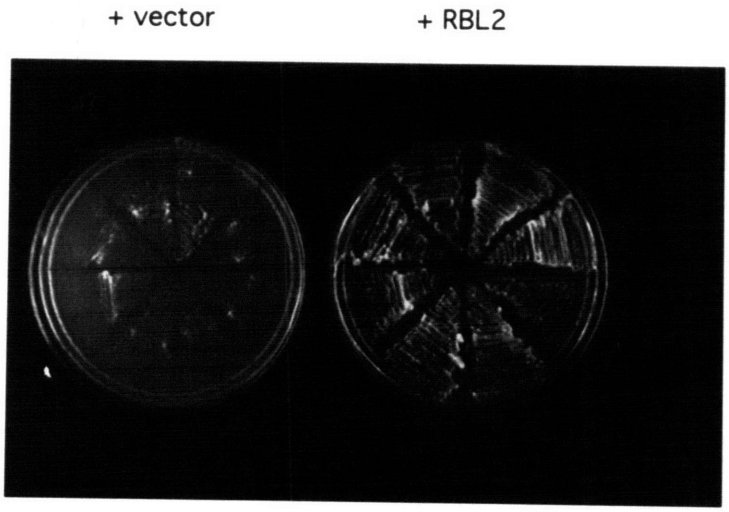
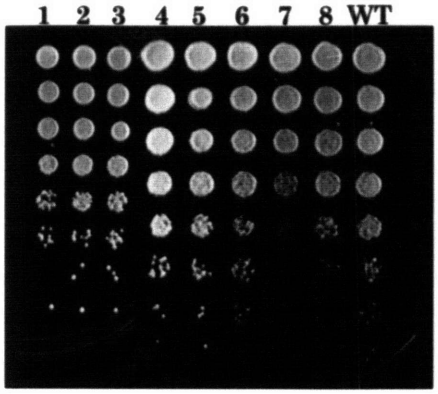
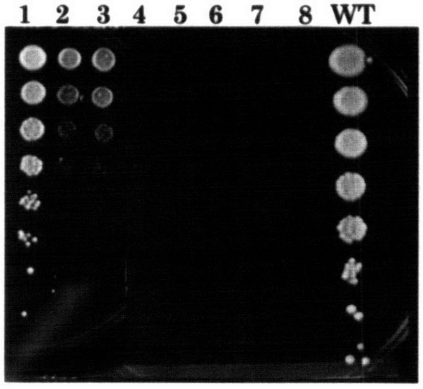

+ vector
+ RBL2
1
8
2
7
3
6
4
5
5-FOA

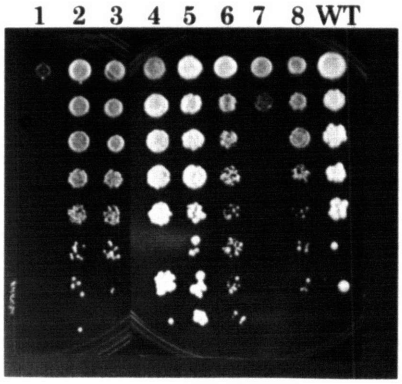
Figure 4-2. Conditional phenotypes of mutants synthetic lethal with Δ*rbl2*. The eight strains (from Figure 4-1) that are synthetic lethal with Δ*rbl2*. are replica plated to plates containing 20 μg/ml benomyl or rich plates at 30°C or 37°C. Each column is a serial dilution beginning at 10^8 cell/ml and decreasing by 1/4s. 1 is temperature sensitive, 2-6 and 8 are benomyl supersensitive and 7 is temperature sensitive and benomyl supersensitive. "WT" is a wild type haploid (FSY183) for comparison.



30°



benomyl 20ug/ml



37°

Figure 4-3. *rks1-1* is not rescued by *TUB1*.

We transformed *rks1-1* cells with a LEU vector backbone or containing *TUB1* (pRB539). We streaked colonies to plates containing 5-FOA. The background is high, but the presence of *TUB1* does not increase viabililty.

rks1-1

SC 5-FOA

Phenotype of *rks1-1* cells

rks1-1 cells fail to grow at 37° C and are slow growing at 30°C. We observe these defects both on plates and in liquid media. When monitored over a time course after a shift to the non-permissive temperature, an asynchronous population of *rks1-1* cells continues to divide for 6 hours, or approximately 2 cell doublings, before ceasing to increase in cell number (Figure 4-4). Subsequently, the cells do not increase in number over more than 24 hours.

Cultures of the *rks1-1* mutant at elevated temperatures contain a significant increased proportion of cells with defective microtubules, as scored by localization of anti-tubulin antibodies (Figure 4-5). We see a more modest defect at the semi-permissive temperature (30°C) than at the non-permissive temperature (37°C). In addition, by phase microscopy we observe a shift to unbudded cells (Figure 4-6). In addition, a large number of tiny cells accumulate (data not shown). These cells are roughly half the size of wild type cells and exhibit all stages of buddedness.

Suppression of *rks1-1*

To clone *rks1*, we transformed a *Saccharomyces cerevisiae* genomic library into a derivative of the original isolate. We selected for survivors at 37°C and obtained five colonies. By restriction map, sequencing and computer analysis,

Figure 4-4. *rks1-1* cells are temperature sensitive.

We shifted *rks1-1* cells containing vector or pJA38 (SUP1) to 37°C at time = 0. We monitored cell number at various times after shift to the non-permissive temperature.

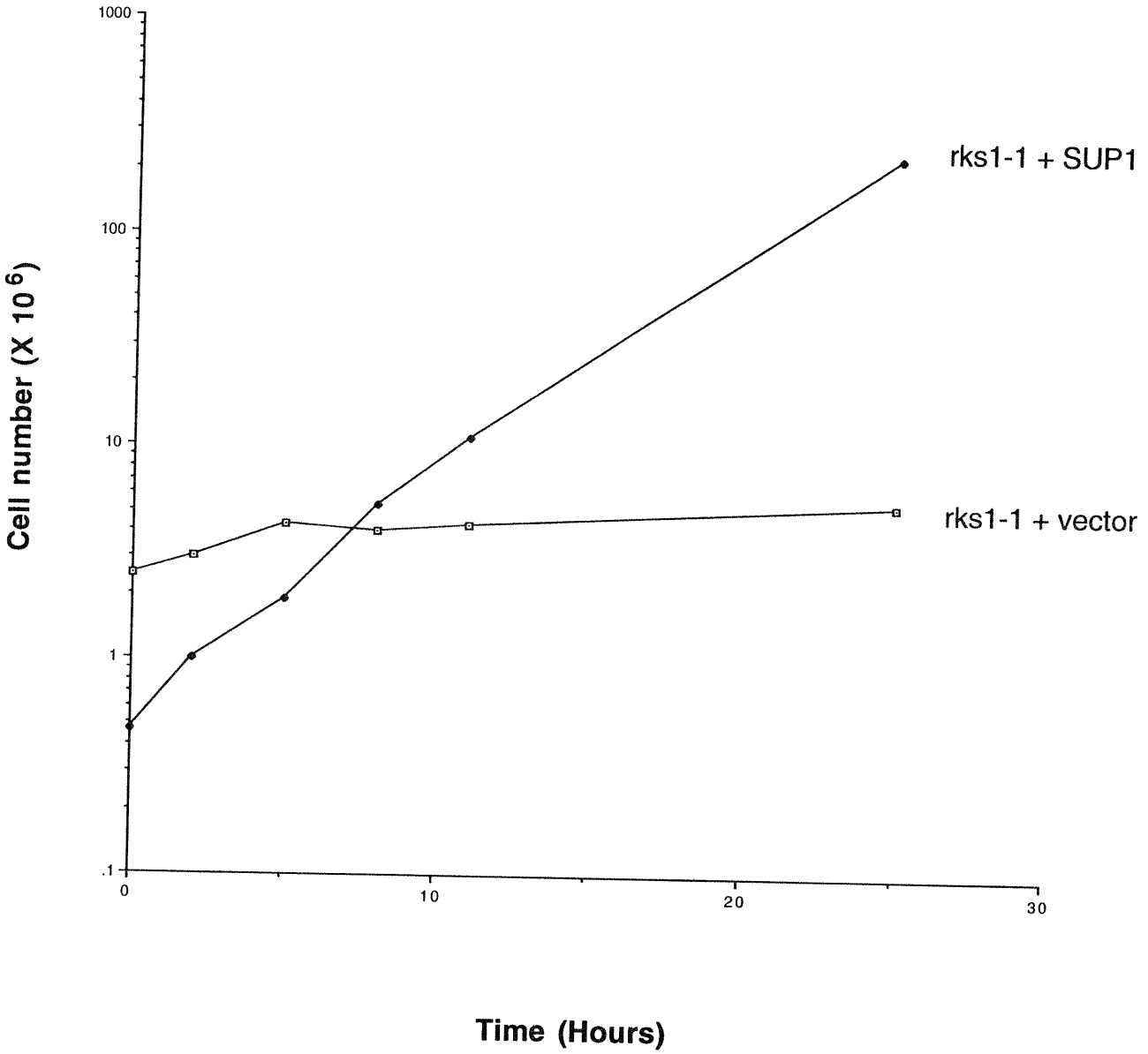
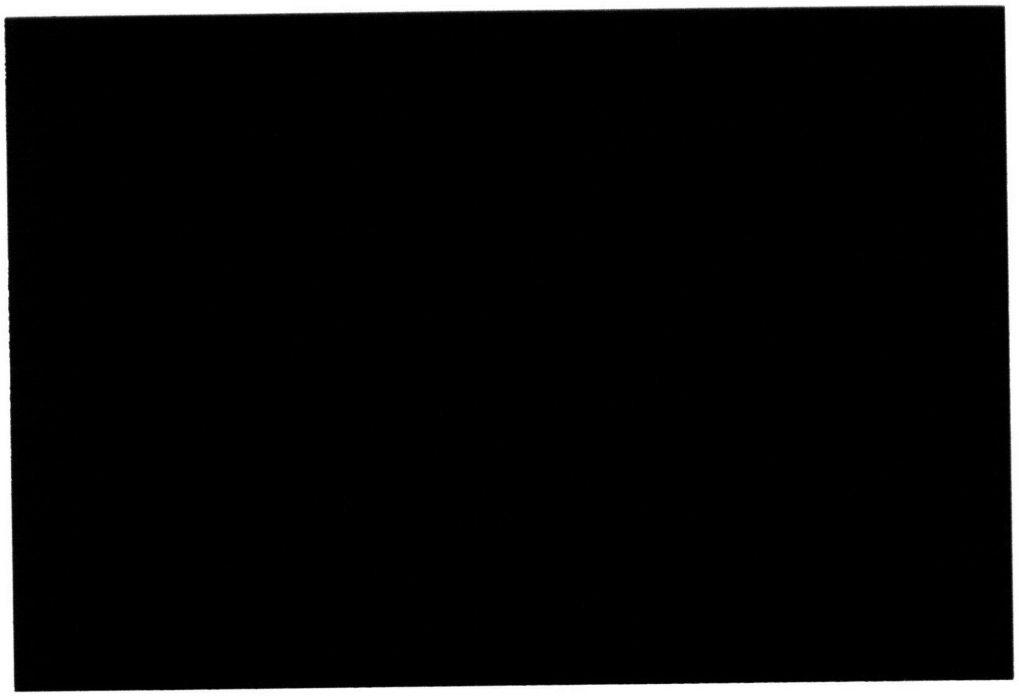

1000
100
10
1
.1
Cell number (X 10 6)
0
10
20
30
Time (Hours)
rks1-1 + SUP1
rks1-1 + vector

Figure 4-5. *rks1-1* cells lose their microtubules at the the non-permissive temperature.

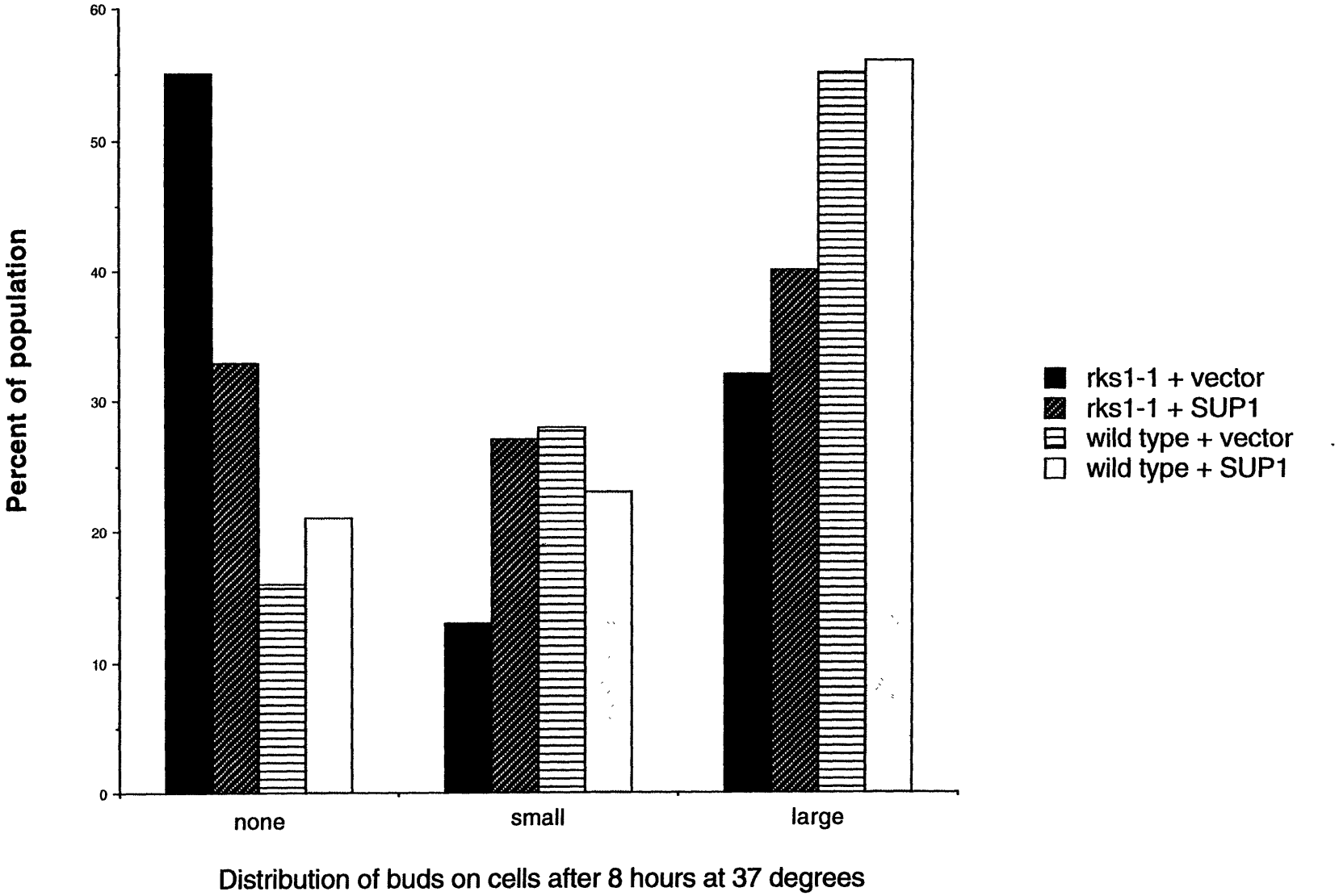
We shifted *rks1-1* cells containing vector (A) or pJA38 (B) to 37°C. After eight hours, we fixed the cultures for immunofluorescence with anti-β-tubulin antibodies.

A.



B.

Figure 4-6. *rks1-1* cells become unbudded at the non-permissive temperature. We shifted *rks1-1* cells containing vector or pJA38 (*SUP1*) and wild type cells containing vector or pJA38 (*SUP1*) to 37°C. After eight hours, we scored cells as containing none, small or large buds.


Percent of population
60
50
40
30
20
10
0
none
small
large
rks1-1 + vector
rks1-1 + SUP1
wild type + vector
wild type + SUP1
Distribution of buds on cells after 8 hours at 37 degrees

we conclude that we have identified two distinct loci, represented respectively by two (*SUP1*) or three (*SUP2*) overlapping inserts (data not shown).

We subcloned a DNA fragment containing only one predicted open reading frame (*SUP1*, Figure 4-7) and find that it is sufficient for complementation of the temperature sensitivity at 37° (Figure 4-4), slow growth at 30° and synthetic lethality with Δ*rbl2* of *rks1-1* cells (Figure 4-8). It encodes a protein of predicted molecular weight 102 kD with no homology to anything in the database, including Rbl2p.

A second open reading frame (*SUP2*) also rescues the temperature sensitivity at 37° and synthetic lethality with Δ*rbl2* of *rks1-1* cells (A. Smith and F. Solomon, unpublished observations). *SUP2* has previously been cloned as *SSD1* (Sutton et al., 1991), *SRK1* (Wilson et al., 1991) and *SSL1* (Costigan et al., 1992) . *SRK1* was isolated as a low-copy suppressor of an allele of the low-Km cyclic AMP phosphodiesterase (*pde2*) and was found to also suppress an *ins1* mutation that arrests with unreplicated DNA and unduplicated SPBs (Wilson et al., 1991. *SSD1* was cloned as a locus that affected the phenotypes of deletions of either *SIT4* (protein phosphatase) or *BCY1* (regulatory subunit of the cAMP-dependent protein kinases) (Sutton et al., 1991) . *SSL1* was isolated as a low copy suppressor of a deletion of *SLK1*, a gene that was

Figure 4-7. Predicted amino acid sequence of Sup1p.

This sequence was generated by the sequencing project and identified as ORF YD9813.05c.

MSISETPHNK SQGLQKAAGR PKIVVPEGSP SRNSDSGSFT IEGDTSLNDD

51 LLSISGSVTP RARRSSRLSL DSITPRRSFD SRTLSVANSR SFGFENETH

101 GSMDFSPLGN NSIYEIVMNT RRKNWLNYPT VADIPQVSLS KNDLDDHWKT

151 HVIEYVKNIK SDYQIFQSTN NIRNMNQMEQ LKELREGENM HEESFEANLR

201 QGDAELINSI PDFYFSDKFQ LDNPRTFHKVLDAIDLFLTK LDMKRQAERD

251 EAFSELRDRL NDFLDIVETL LVTEISKSSH KFFHALSEVD NIQKRALDTM

301 SELKELAQNI KTIDAENIRK KISHLEMIFK RKNVEKLEQG LLQAKLVLNK

351 TDECKSMYEE NKLDNCLELI KSIDYLIKGD DSINEDVQSW TRCWPYKLSN

401 LRTIPALSAT REFLTNMKIE IGGKFSLQLS ILLIDDLRSF CKSIKPKETL

451 HRIQTGSNDK KQTIFTDNFS SKITELIVRL NRCEELTSAF DLYREKSITE

501 LKSIIKIYLP TENAHADNNH DEKHLNNGST SGSKLSRLIK EQTPAEFQSM

551 LVNIFTHALE ALRRLYGHQK LLLDISLNEL ASVKSPNENQ HNMITQLDIR

601 TGINEIIRII QLRTGKIIAV RRELNLSLRY DYFLKFYAIC VIFIQECEVL

651 SGEFLTKYLS NVLASQIKHY ANAQSSKNYR NIKKKIDAEE WIPYIVDSSI

701 QSDVNDIVSS IDIDPLSWTT ILDMVGGSHD CENGRSEDKE KDEGNETYQG

751 HRKSVVVGDK TFVASSSLLA TIEVIKELMV LSINLPSIYL SNFEKLCYDA

801 LQYYNSSAMA SVTQPGNSLL KTGRNLSIMG ESLDCLAEFV IIVQRFYQRL

851 SNSNRDFEPF DASHYTTLLG QFQASSNKIY MANAPPPPV*

Figure 4-8. *SUP1* rescues the synthetic lethality of *rks1-1.*

We transformed *rks1-1* cells with pJA38 (*SUP1*) or vector alone and streaked colonies to plates containing 5-FOA.

SC 5-FOA

isolated as synthetic lethal with *SPA2* and has sequence similarity to protein kinases (Costigan et al., 1992) . Sup2p encodes a protein of predicted molecular weight 140 kD and contains glutamine and asparagine rich regions and putative cAMP kinase phosphorylation sites, but has no homology to Rbl2p. Its identification in several screens related to kinases/phosphatases raises the possibility that phosphorylation is playing a role in the assembly pathway of β-tubulin. However, SUP2 may be acting non-specifically rather than proximally to RBL2, because it appears as a suppressor in so many screens.

Phenotype of Δ*sup1* cells

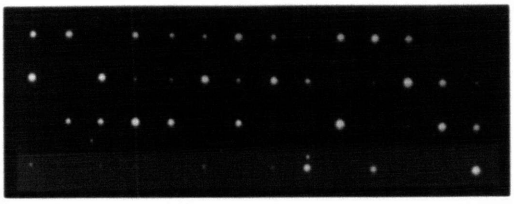
We constructed a complete disruption of the *SUP1* open reading frame (Figure 4-9). Spores containing this deletion appear viable, but slow growing. However, out of five heterozygotes we examined, only three produced tetrads with clean two to two segregation patterns by Southern blot analysis (Figure 4-9B). The remainder produced offspring with excess undisrupted *SUP1* alleles. We have analyzed haploids from heterozygotes which appeared to produce only clean patterns of segregation.

Δ*sup1* cells are temperature, cold and benomyl sensitive (Figure 4-10A). When monitored over a time course after a shift to the non-permissive

Figure 4-9. *SUP1* is not an essential gene.

We created a complete disruption of the *SUP1* open reading frame. (A) After dissecting heterozygotes, we find tetrads are 4:0 for life, although roughly 2:2 for small size. The small colonies correspond to Leu+ colonies. (B) Analysis of four tetrads by Southern analysis shows abnormalities in some cases. The first two tetrads (A-D) are from heterozygote #12 and contain excess wild type bands. The second two tetrads are from heterozygote #17 and contain 2 wild type: 2 disrupted alleles.

A



B

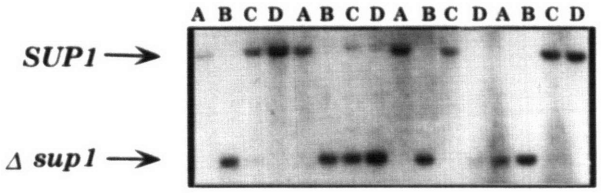
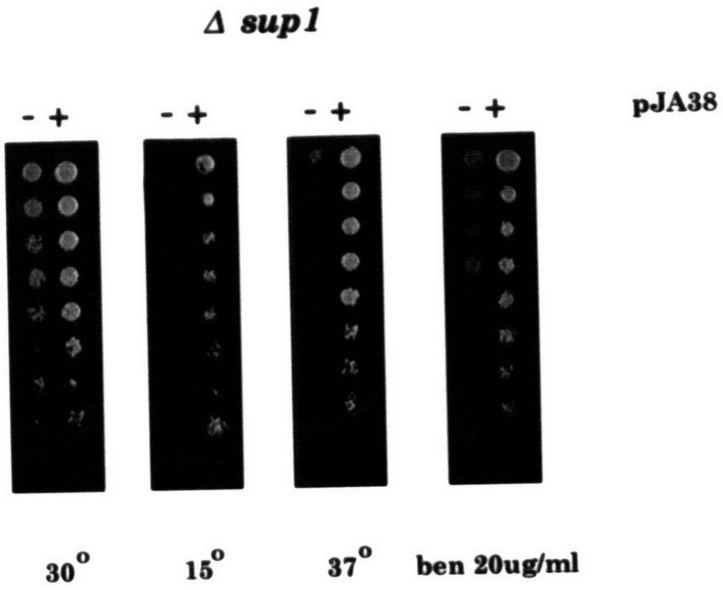
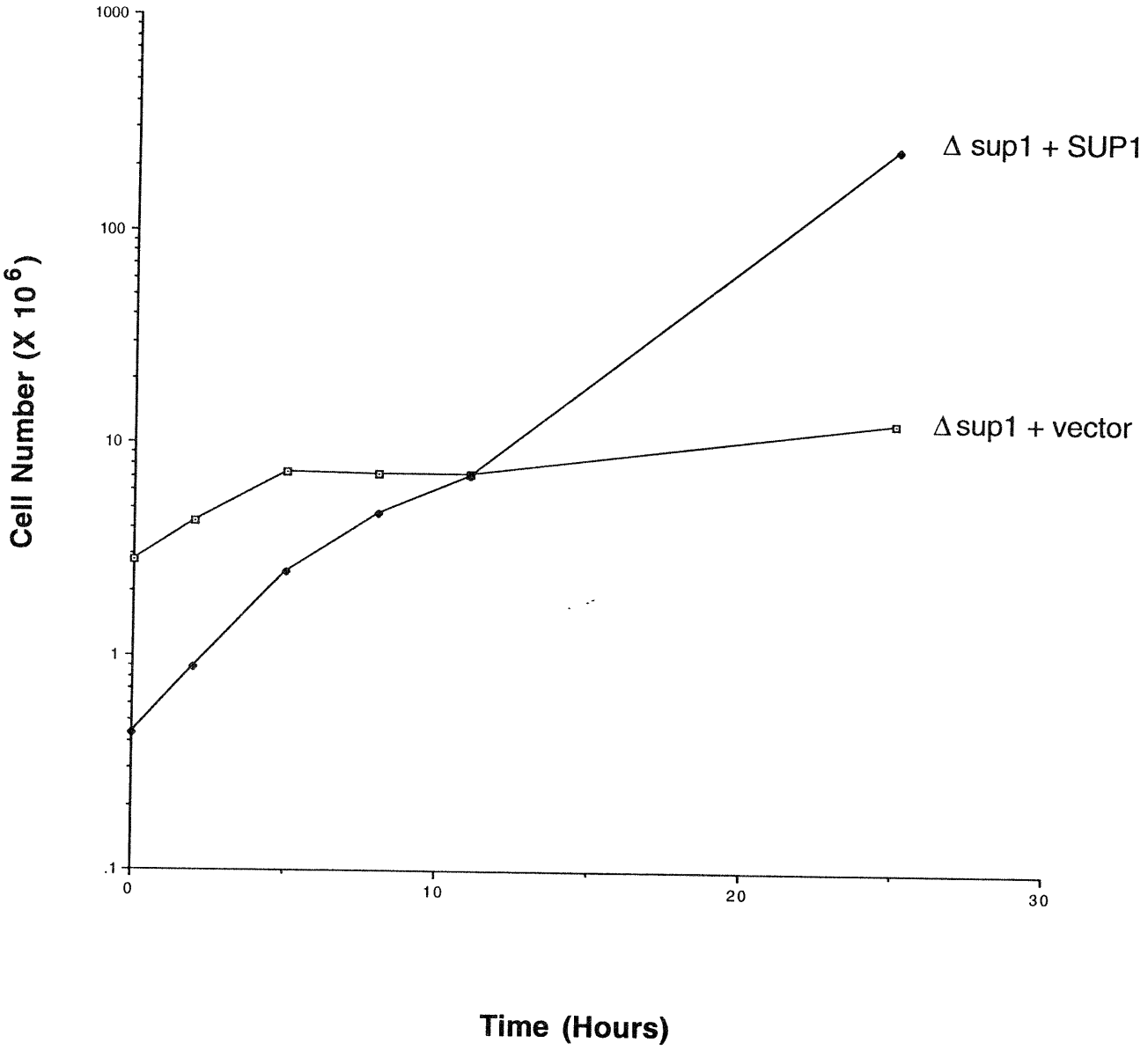
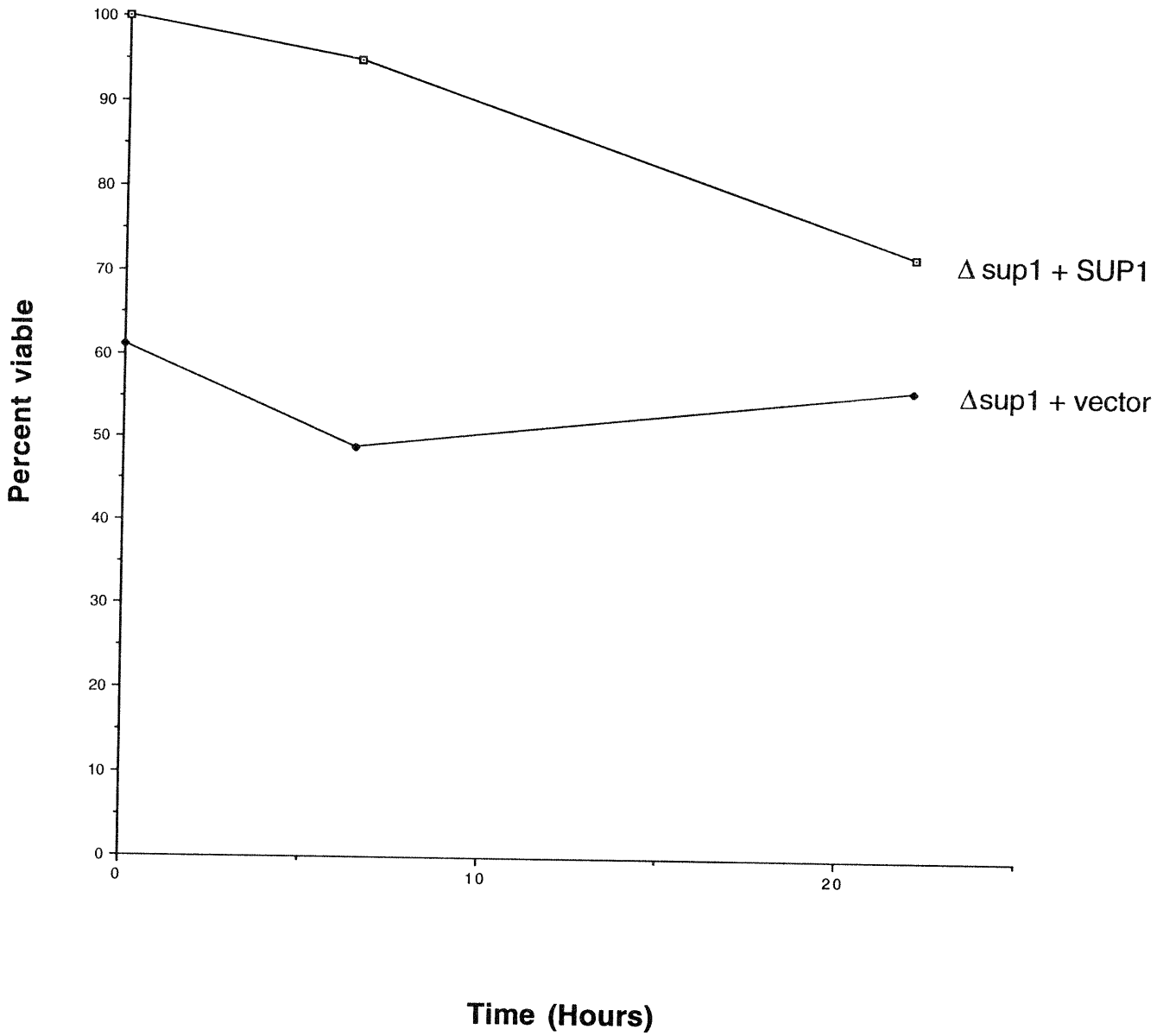


Figure 4-10. Δ*sup1* cells exhibit conditional defects.

We transformed Δ*sup1* cells with a vector backbone (-) or pJA38 (+ *SUP1*). (A) We replica plated these strains to rich plates at 30°C, 15°C or 37°C or to plates containing 20 μg/ml benomyl. Columns are serial dilutions of cells beginning at 10^7 cells/ml and descending by halves. (B) We shifted the strains to 37°C at time = 0. We monitored cell number at various times after shift to the non-permissive temperature. (C) We quantitatively plated cells after various times at 37°C and grew them at 30°C to determine cell viability. Δsup1 cells have decreased viability even when grown at their "permissive" temperature (T=0), but they do not become less viable at the non-permissive temperature.


Δ sup1
- +
- +
- +
- +
pJA38
30°
15°
37°
ben 20ug/ml


1000
100
10
1
.1
Cell Number (X 10^6)
0
10
20
30
Time (Hours)
Δ sup1 + SUP1
Δ sup1 + vector


Percent viable
100
90
80
70
60
50
40
30
20
10
0
0
10
20
Time (Hours)
Δ sup1 + SUP1
Δsup1 + vector

temperature, an asynchronous population of Δ*sup1* cells continues to divide for 6 hours, or approximately 3 cell doublings, before ceasing to increase in cell number (Figure 4-10B). However, the arrested cells do not appear to lose viability while its cell number remains static. Even after 22 hours at 37°C, the population is alive if returned to permissive conditions (Figure 4-10C). Either *SUP1* or *SUP2* rescue the temperature sensitivity of Δ*sup1* cells (Figure 4-11).

When we analyze the status of Δ*sup1* microtubules, we observe a similar but less severe effect than in *rks1-1* cells. After shifting cells to the non-permissive temperature, they lose tubulin structures (Figure 4-12) although a larger percentage of the population maintains assembled microtubules than do *rks1-1* cells. Furthermore, like *rks1-1* cells, Δ*sup1* cells accumulate unbudded (Figure 4-13) and tiny cells at the non-permissive temperature.

Surprisingly, Δ*sup1* is not synthetic lethal with Δ*rbl2*. While this cross was subject to chromosomal abnormalities leading to excess wild type *SUP1* alleles (as above), we did recover haploids with only Δ*sup1* and hence the negative result appears to stand.

Figure 4-11. Δ*sup1* cells are rescued by *SUP2*.

We streaked out Δ*sup1* cells containing vector plus *SUP2* (pJ3) or vector alone and grew them at 37°C.

Δ *sup1*

SUP2

vector

37 °

Figure 4-12. Δ*sup1* cells lose their microtubules at 37°C.

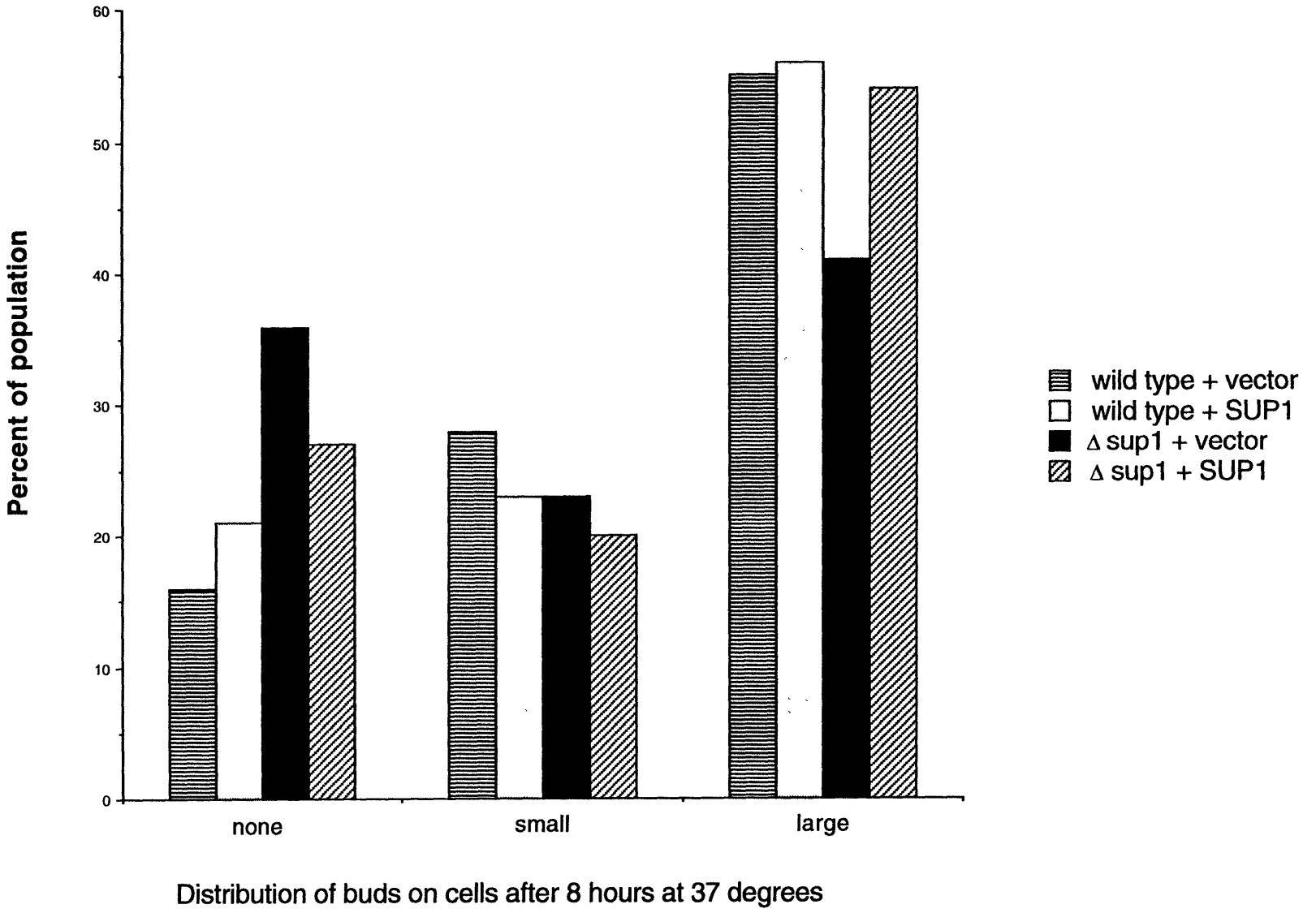
We shifted Δ*sup1* cells containing vector (A) or pJA38 (B) to 37°C. After eight hours, we fixed the cultures for immunofluorescence with anti-β-tubulin antibodies.

A.

B.

Figure 4-13. The proportion of unbudded cells increases in a Δ*sup1* population.

We shifted Δ*sup1* cells containing vector or pJA38 (*SUP1*) and wild type cells containing vector or pJA38 (*SUP1*) to 37°C. After eight hours we scored cells as containing none, small or large buds.


Percent of population
60
50
40
30
20
10
0
none
small
large
wild type + vector
wild type + SUP1
Δ sup1 + vector
Δ sup1 + SUP1
Distribution of buds on cells after 8 hours at 37 degrees

Overexpression of *SUP1*

We constructed SUP1 under the control of the GAL1-10 promoter. The over-production of SUP1 is not lethal at any temperature tested (data not shown). Furthermore, it does not appear to lead to altered growth on benomyl (data not shown). Finally, *SUP1* does not act as a RBL, that is it does not rescue the lethality of excess ß-tubulin (Figure 4-14). We have not demonstrated by Northern or Western analysis that overexpression does in fact occur in these strains.

Epitope tagged *SUP1*: physical associations

We constructed triple and hextuple hemagglutin (HA) epitope tagged *SUP1* (M. Magendantz and F. Solomon). The tags are inserted at the extreme C-terminus of the *SUP1* coding sequence. The construct preserves the endogenous *SUP1* promoter and is present on a *CEN* plasmid in a Δ*sup1* strain. These versions of *SUP1* are in fact expressed (Figure 4-15), and they complement the temperature sensitivity of the deletion (Figure 4-16), suggesting they retain functional competence.

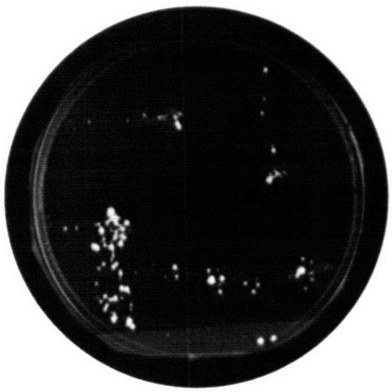
We examined the possibility that Sup1p might associate with Rbl2p, as assayed by its presence on nickel beads bound by Rbl2-His6p. We harvested proteins from cells whose sole source of Sup1p is HA-tagged (see above). We

Figure 4-14. *SUP1* does not suppress overexpression of β-tubulin.

We overexpressed *SUP1* (pJA44) or vector alone in JAY47 (containing GAL1-10 promoted *TUB2*) by streaking cells on plates containing galactose.

JAY47

vector



pJA44

SC-Galactose

Figure 4-15. Epitope-tagged *SUP1* is expressed.

We harvested protein from Δ*sup1* cells containing pJA38 (A), pSET1(B) or pSET3 (C). We loaded 2X and 1X loads of protein on a 7.5% acrylamide for Western analysis with antibody 12CA5. Note that the hextuple epitope (pSET1) is more easily detected that the triple epitope (pSET3).

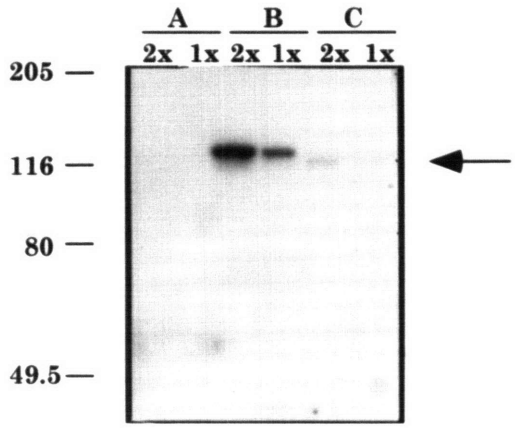
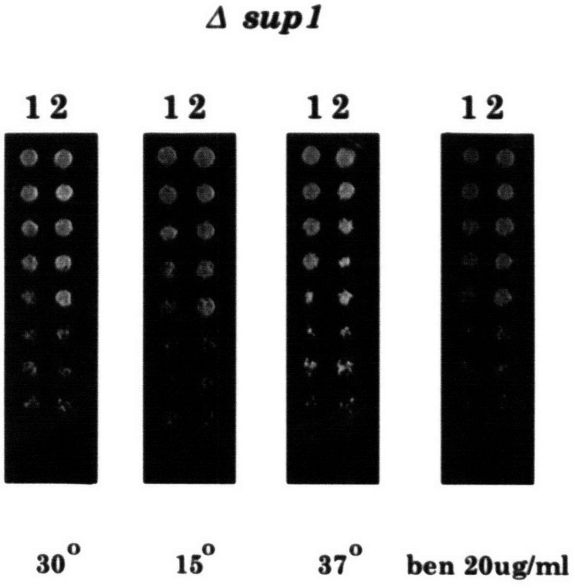

A
B
C
2x 1x 2x 1x 2x 1x
205
116
80
49.5

Figure 4-16. Epitope-tagged *SUP1* rescues Δ*sup1* cells.

We transformed Δ*sup1* cells with pSET1 (1) or pSET3 (2) and replica plated these strains to rich plates at 30°C, 15°C or 37°C or to plates containing 20 μg/ml benomyl. Columns are serial dilutions of cells beginning at 10^7 cells/ml and descending by halves. This figure may be directly compared to Figure 4-10A.


Δ sup1
1 2
1 2
1 2
1 2
30°
15°
37°
ben 20ug/ml

incubated this extract with recombinant Rbl2(His6)p and nickel beads. By performing Western analysis with 12CA5, a monoclonal antibody directed against the HA tag, we find that Sup1p does associate with Rbl2p by this criterion (Figure 4-17).

Finally, we attempted to localize Sup1p by immunofluorescence. Again, we used the monoclonal antibody 12CA5 to detect Sup1(HA)p. To date, we see no evidence of specific localization at either 30° or 37°C (data not shown).

Allelism of *rks1-1* and Δ*sup1*

Do either *SUP1* or *SUP2* encode wild type *RKS1*, or are they second site suppressors? *rks1-1* and Δ*sup1* share many phenotypes. They differ in cold and benomyl sensitivity (only Δ*sup1*) and in synthetic lethality with Δ*rbl2* (only *rks1-1*). As mentioned above, *rks1-1* and Δ*sup1* are each difficult to manipulate genetically. Therefore, we have not yet completed interpretable, true allelism analysis. So far, indirect tests are consistent with *rks1-SUP1* allelism or close genetic interaction. (1) As stated above, *SUP2* complements both *rks1-1* and Δ*sup1*. (2) Diploids containing *rks1-1* and Δ*sup1* fail to complement for temperature sensitivity (Figure 4-18). Therefore, *rks1-1* and Δ*sup1* represent one locus or they are unlinked non-complementers. (3) If we disrupt *SUP1* in *rks1-1* haploids, the cells lose their synthetic lethality with

Figure 4-17. Sup1(HA)p associates with Rbl2(His)p.

We harvested protein from Δ*sup1* cells containing pJA38 (-) or pSET1 (+) and incubated it with recombinant Rbl2(His6)p and nickel slurry. After elution, we analyzed the samples by Western blot with the 12CA5 antibody.

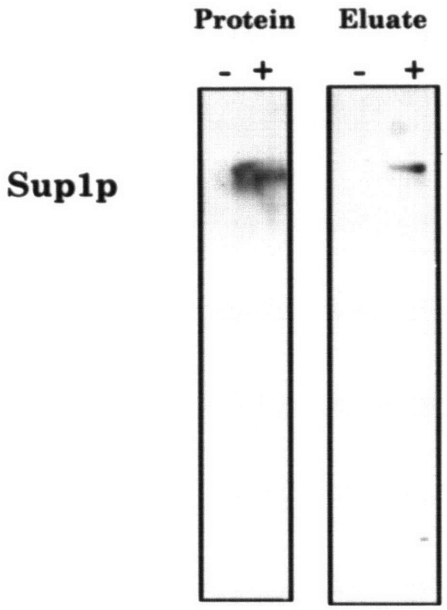

Protein
Eluate
- +
- +
Sup1p

Figure 4-18. *rks1-1* and Δ*sup1* do not complement for temperature sensitivity.

We crossed *rks1-1* haploids with wild type (WT) or Δ*sup1* haploids. We streaked the diploid products to plates at 37°C.

rks1-1 **x**

WT

Δ *sup1*

37 °

Δ*rbl2* (Figure 4-19). This result is consistent with the *rks1-1* allele having been replaced by a disruption of the locus, which we showed does not have a rks phenotype. Alternatively, *rks1-1* might require wild type *SUP1* for its synthetic lethality event.

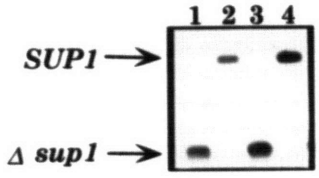
In order to confirm the allelism, we are back-crossing rks1-1, so that we can perform linkage analysis. In addition, we are using gap repair to analyze the status of SUP1 in the rks1-1 strain.

DISCUSSION

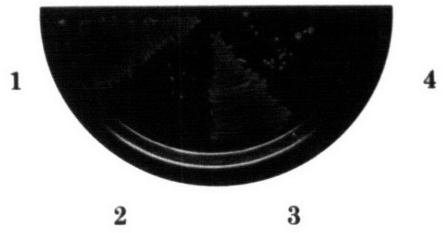
We have identified mutations that are synthetic lethal with the deletion of *RBL2*. Seven of them are alleles of *TUB1*, and may be useful for further analysis. Conveniently, they are chromosomal alleles because they were generated through *in vivo* mutagenesis. This contrasts with the panel of *TUB1* mutants generated by *in vitro* mutagenesis (Schatz et al., 1988) , which are plasmid borne and not generally recoverable in integrated form (D. Botstein, personal communication). In addition, these alleles may have a common defect, such as in dimerization. Therefore, we might gain an

Figure 4-19. The disruption of *SUP1* in *rks1-1* cells relieves their synthetic lethality with Δ*rbl2*.

We transformed *rks1-1* cells with a DNA construct for the *LEU2*-marked disruption of *SUP1*. We analyzed the Leu+ transformants by Southern blot for the presence of disrupted *SUP1*. We also checked the Leu+ transformants for the ability to grow on plates containing 5-FOA. 1 and 3 are disrupted for *SUP1* and can now grow on plates containing 5-FOA. 2 and 4 remain *SUP1*+ and unable to grow on plates containing 5-FOA.



rks1-1



5-FOA

understanding of specific aspects of tubulin activity through a structure-function analysis of these alleles.

We identified a non-tubulin mutant that is synthetic lethal with the *RBL2* knockout and have provisionally named it *rks1-1* (*RBL2* Knockout Synthetic lethal). The fact that this screen has identified only one new interacting locus is more striking than alarming. It seems likely that only a small number of genes may answer this screen.

rks1-1 is itself temperature sensitive and loses its microtubules at the non-permissive temperature. *SUP1* probably encodes the wild type version of *rks1-1* and its deletion is also temperature sensitive for viabililty and microtubules. Sup1p physically interacts with Rbl2p, but it is not itself a RBL. How then might Sup1p fit into the β-tubulin assembly pathway? Perhaps Sup1p forms a complex with Rbl2p necessary for active β-tubulin binding (model one). Alternatively, it could bind α-tubulin monomer and then associate with Rbl2p to promote dimerization (model two).

No matter what the mechanism of action, something must explain different between the deletion and the allele of *SUP1/RKS1*. Under model one, it might be that the Rbl2p-Sup1p complex is overlapped in function by a second

functionally homologous activity. In the absence of Rbl2p or Sup1p or both, the second activity may largely be able to compensate. However, the rks1-1p might be able to poison the action of the second, either by interacting directly with β-tubulin or with the second activity. Although *SUP1* is not a RBL perhaps it does possess some ability to bind directly to β-tubulin, an ability that is increased in the mutant version of the protein. Or, perhaps the second activity is composed of weakly related Rbl2p and Sup1p homologs, so rks1-1p is able to interact with the RBL2 homolog and poison its activity. Under model two, rks1-1p could interact with α-tubulin aberrantly and interfere with dimerization or polymerization. The sequence alteration of the *rks1-1* allele may be informative. If *SUP1* does not encode the wild type version of *rks1-1*, we need to sort out the more complicated interactions between at least three gene products (Rbl2p, Rks1p and Sup1p).

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CHAPTER 5

CONCLUSION

Microtubule assembly occurs from a pool of α-β-tubulin heterodimers. Heterodimers themselves are the result of an assembly pathway involving folding and dimerization. We have characterized Rbl2p, a yeast protein that binds to a monomeric form of β-tubulin. We identified *RBL2* in a screen for yeast genes that when overexpressed can rescue cells from the lethality caused by an excess of β-tubulin. *RBL2* is not essential for vegetative growth, but changes in its level affect microtubule pathways and meiosis. *RBL2* over-expression, like the over-expression of α-tubulin, confers resistance to benomyl. Both the deletion and the over-expression of *RBL2* are synthetically lethal with specific mutant alleles of α-tubulin. Rbl2p is structurally and functionally homologous to murine cofactor A. Cofactor A was identified as a polypeptide involved in tubulin folding and dimerization in an *in vitro* assay (Gao, et al., 1994). Our results suggest that Rbl2p interacts with monomeric ß-tubulin as a distinct step in an assembly pathway, independent of the chaperonin-mediated folding. Furthermore, Rbl2p appears to recognize a nearly native form of β-tubulin, a conformation very similar to that which binds α-tubulin. Finally, we have identified an allele of a yeast gene, *rks1-1*, that genetically interacts with *RBL2* and exhibits a microtubule phenotype. The product of a suppressor of *rks1-1*, *SUP1*, physically interacts with Rbl2p and also exhibits microtubule phenotypes. Our results suggest that we have begun to define a group of tubulin associated proteins that may serve an

analogous role to scaffolding proteins in other morphogenetic pathways - participating in intermediates but not present in the stucture itself.

G-actin binding proteins

Actin folds in vitro by the action of the TCP1 complex in the absence of any cofactors (Gao et al., 1992). However, there exist proteins that bind to monomeric actin, such as profilin and β-thymosin. β-thymosin seems to act as a monomer sequestering protein, maintaining a pool of unpolymerized actin (Sanders et al., 1992). Profilin has been reported to promote microfilament assembly when overexpressed in animal cells (Finkel et al., 1993), but to lead to depolymerization when microinjected (Cao et al., 1992). However, the microinjection of a profilin-actin complex led to actin polymerization and membrane ruffling (Cao et al., 1992). It localizes in the cell to regions of active microfilament assembly (Bub et al., 1992) and is associated with polymerizing actin tails of Listeria (Theriot et al., 1994). In vitro, profilin activity is affected by the presence of other actin binding proteins such as β-thymosin and gelsolin (Pantaloni and Carlier, 1993). Profilin also acts to promote nucleotide exchange on actin (Goldschmidt-Clermont et al., 1992).

Because significant amounts of tubulins are not detected in monomeric form by native gel, it is unlikely that monomer sequestration is an important form

of regulation of microtubule assembly. Rbl2p could promote assembly with a mechanism similar, or not, to that of profilin. Rbl2p could play a role in nucleotide exhange on β-tubulin, as does profilin for actin. On the other hand, profilin forms a complex with the basic subunit of the polymer and therefore can be involved with the substrate for addition at the growing end of the filament. The β-tubulin associated with Rbl2p, however, is almost certainly not the substrate for polymer addition. The possibility remains that Rbl2p-β-tubulin interacts with γ-tubulin or another factor as part of a nucleation reaction. This model is not readily consistent with the ability of overexpressed *RBL2* to suppress overexpressed *TUB2*, if we assume that β-tubulin's lethal event is binding to the SPB (see Chapter 2).

Why does Rbl2p bind to β-tubulin?

There are a variety of proteins that interact with microtubules and/or affect microtubule structure and stability (see Introduction). Some of these may have binding sites on β-tubulin, but would interact in the context of the dimer or polymer. Rbl2p interacts specifically with monomeric β-tubulin. Another candidate for a β-tubulin monomer binding protein is Cin1p. Tian et al. recently described a "pathway to correctly folded β-tubulin" that required three cofactors after the TCP1 complex to make β-tubulin competent for

exchange into dimer (Tian et al., 1996) . In their current model, Rbl2p (cofactor A) is a side pathway for newly synthesized β-tubulin, whereas cofactors D, E, and C are all required for the conversion of β-tubulin to an exchangeable form. Cofactor D/Cin1p formed a complex with β-tubulin. Although cofactor D is required in this *in vitro* assay, it is not an essential gene (Stearns et al., 1990; Hoyt et al., 1990) . As discussed in chapter 4, the reason for this discrepancy may be the presence of overlapping activities. We find Δcin1 Δrbl2 double mutants sick or dead (J. Fleming, J. Archer and F. Solomon, unpublished observations). However, the pathway may be quite a bit more complicated. For instance, *CIN2* and *CIN4* were isolated with *CIN1* and all three share many phenotypes. We find that mutants/nulls of each are synthetic lethal with the overexpression of *RBL2* and sick or dead with Δ*rbl2* (J. Fleming and F. Solomon, unpublished results). The roles of Cin2p and Cin4p in the assembly pathway remain obscure at this point. In addition, we find that Sup1p interacts with Rbl2p (Chapter 4). We are now addressing the effect this binding has on Rbl2p's interaction with tubulin, as well as determining whether Sup1p interacts directly with either α- or β-tubulin.