Characterization of the Alpha- and Beta-Tubulin Polypeptides in 
*Saccharomyces cerevisiae*

by

Vida Praitis
B.A., Biology. Swarthmore College, 1988

Submitted to the Department of Biology in Partial Fulfillment of the Requirements for the Degree of

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Characterization of the Alpha- and Beta-Tubulin Polypeptides in *Saccharomyces cerevisiae* by Vida Praitis

Submitted to the Department of Biology on May 5, 1995 in partial fulfillment of the requirements for the Degree of Doctor of Science in Biology

Abstract:

Microtubules, involved in a number of critical, diverse cellular structures and functions, are composed primarily of two related but non-identical, highly conserved subunits, alpha- and beta-tubulin. The precise secondary and tertiary interactions of the tubulin subunits are still poorly understood, likely because native alpha- or beta-tubulin has not been purified in large enough quantities to perform structural or biochemical analysis. Crystal structures, which would provide extensive structural information about these molecules and their interactions, have not been reported. I sought to characterize the alpha- and beta-tubulin polypeptides using two approaches, one genetic and one biochemical.

E. Raff's laboratory characterized a series of mutations in the testes-specific beta-tubulin of *D. melanogaster*. Strains homozygous for B2t8 exhibited an intriguing phenotype. All microtubule structures were disrupted, with S- or U-shaped, rather than O-shaped microtubule cross-sections. The phenotype suggested a defect in packing within the microtubule polymer. Sequence analysis revealed a glutamic acid to lysine substitution at highly conserved position 288. I generated the same mutation in the sole beta-tubulin of *S. cerevisiae*. Phenotypic analysis revealed no defects in growth at several temperatures, mating, sporulation, or germination. The only phenotype was a slight alteration in the sensitivity to the anti-mitotic drug benomyl. These results demonstrate that microtubules are differentially susceptible to in vivo growth conditions or alterations in the beta-tubulin primary sequence.

To examine the properties of the individual tubulin subunits biochemically, we developed a procedure to enrich for alpha- or beta-tubulin in the absence of its heterodimeric partner. Co-immunoprecipitated alpha- and beta-tubulin dissociated when exposed to low concentrations of non-ionic detergents. GTP inhibited the detergent-mediated separation of tubulin heterodimer. Relatively pure alpha- and beta-tubulin was tested for its ability to bind GTP, using a photo-cross-linking assay. Native levels of GTP binding could be restored in the eluates when both tubulin subunits were present in equimolar amounts, demonstrating both chains contribute to tubulin GTP binding.

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Chapter One: Introduction
Introduction

Microtubules are involved in a vast number of critical and complex cellular functions. The biochemistry and structure of the tubulin heterodimer and microtubules have been examined for more than thirty years, yet much remains to be explored. This introduction examines a number of hypotheses and experiments from the literature exploring tubulin biochemistry and structure. The intention is to provide a historical and experimental framework for the research described in later chapters of this dissertation.

Microtubule Function and Theory

Microtubule Function

Microtubules are polymeric structures, composed primarily of alpha- and beta-tubulin, present in a number of cellular structures. They are involved in several critical cellular processes which can be broadly characterized in four ways. First, they provide structural rigidity required to specify a cell's characteristic shape, including that of highly specialized cells such as neurons and erythrocytes. Second, microtubules are required for the structural rigidity of cellular structures, such as the mitotic spindle. Third, they provide a structural matrix upon which critical materials are transported within cells. Finally, they produce structures required for cell motility, such as sperm axonemal complexes and flagella.

The vast functional capacity of microtubules requires they have several almost contradictory intrinsic properties. First, within a single microtubule structure a number of different microtubule-mediated events occur simultaneously. Microtubules must either be multi-functional as a population, or they must contain specialized microtubules within a population, each capable of performing a single or few tasks. Second, microtubule structures reorganize, often quite rapidly, in response to changes in cellular requirements or as cells differentiate. Yet once microtubules are assembled, they must be structurally sound.
The events in one microtubule-mediated event, mitosis, provide a number of good examples of microtubule functional complexity. Microtubules are the primary structural components of the mitotic spindle, responsible for the accurate and reproducible separation of chromosomes during nuclear division. The transition from interphase to mitosis requires a dramatic reorganization of microtubules, necessitating that the microtubules be capable of radically and rapidly reorganizing in response to cellular signals. During mitosis itself, some microtubules reorganize while others appear fairly stable. Within the mitotic spindle, microtubules provide the rigid framework of the mitotic spindle. Other microtubules interact, directly or indirectly, with chromosomal material to align chromosome pairs at the metaphase plate. Only after the correct pairing of the chromosomes, microtubules mediate the poleward separation of chromosomes at anaphase. Within the mitotic spindle, complex microtubule-mediated events are occurring simultaneously.

There are a number of other excellent examples of microtubule functional diversity within a single specialized microtubule structure. Clearly, microtubules are capable of performing a number of varied and complex roles. How these polymers perform these complex tasks has been a critical question in cell biology. The question is complicated by the composition of microtubules; they are primarily composed of two subunits, alpha- and beta-tubulin, which interact to form a heterodimer. Alpha- and beta-tubulin proteins are highly homologous to one another within a species and each is quite similar to alpha- and beta-tubulins across highly divergent species (Little and Seehaus, 1988; MacRae and Langdon, 1989; Raff, et al., 1987). In addition, the basic physical requirements for a microtubule polymer are contained within the tubulin heterodimer, since purified alpha- and beta-tubulin heterodimer has the capacity to polymerize (Lee and Timasheff, 1975). A critical question then arises. How can such a seemingly simple system account for the diversity and complexity in microtubule structure and function?

**Models of Microtubule Functional Characteristics**

Three major models which are not mutually exclusive have emerged to explain the ability of microtubules to participate in a diverse array of structures and functions -- the Multi-tubulin Hypothesis, The Dynamic Instability
Hypothesis, and The Microtubule-associated Protein Hypothesis. I will briefly describe each of these models.

**Multi-tubulin Hypothesis**

The Multi-tubulin Hypothesis, first described prior to the identification and characterization of tubulin primary sequences, proposed that microtubule functional diversity was possible because of the presence of multiple isoforms of the primary subunits of microtubules, alpha- and beta-tubulin (Fulton and Simpson, 1976; Stephens, 1975). A specialized isoform of alpha- and/or beta-tubulin was required for the establishment of a specific cellular microtubule-structure. As a consequence, isoforms of alpha- and beta-tubulin would be spatially and developmentally restricted.

As research on tubulin isoforms advanced, it became clear that each isoform of alpha- and beta-tubulin expressed in a cell co-localized, and was present in both general and specialized microtubule structures (Lewis, et al., 1987). However, expression of some isoforms was restricted to certain cell types. The Multi-tubulin Hypothesis was modified to address these results (Reviewed In: Cleveland, 1987; Joshi and Cleveland, 1990; Katz and Solomon, 1989; MacRae and Langdon, 1989; Solomon, 1991)). The hypothesis contended that only specialized isoforms could participate in all cellular microtubule structures, including specialized microtubule structures such as the erythrocyte marginal band. In contrast, abundant isoforms could participate only in general microtubule structures. Implicit in this model was the notion that conserved regions of alpha- and beta-tubulin primary sequence were required for conserved functions while divergent regions were required for divergent microtubule functions.

There are numerous alternatives to the Multi-Tubulin Hypothesis that explain the presence of spatially and temporally expressed multiple isoforms of alpha- and beta-tubulin. One is that alpha- and beta-tubulin isoforms arose through gene duplication and they diverged slightly in portions of their primary sequence not required for function. Alternatively, isoforms of alpha- and beta-tubulin may be multi-functional and interchangeable, but the genes are not. Specialized 5' untranslated regions may have evolved to spatially or developmentally regulate expression of tubulin isoforms due to tissue- or cell-
specific requirements (Raff, et al., 1987). Finally, it is possible that alpha- and beta-tubulin isoforms diverged because some isoforms, expressed in specialized cells, participated only in specialized microtubule-mediated functions or structures. As a consequence, the tubulin primary sequences required for other, non-specialized microtubule functions diverged because there was no selective pressure to maintain them. The prediction of this model is that the most highly conserved isoforms would be able to participate in most microtubule-mediated functions while the divergent isoforms would be restricted only to roles in the microtubule structures of the cells in which they are normally expressed (Hoyle and Raff, 1990; Little and Seehaus, 1988).

**Dynamic Instability Hypothesis**

A second major model designed to address questions of microtubule dynamics was the Dynamic Instability Hypothesis. The Dynamic Instability Hypothesis arose because of discrepancies between data examining the kinetics of microtubule polymerization and data from *in vivo* measurements of microtubule polymerization dynamics (Kirschner and Mitchison, 1986; Mitchison and Kirschner, 1984; Mitchison and Kirschner, 1984). At equilibrium, the rates of polymerization and depolymerization should be dependent upon one another. Equilibrium models using experimentally measured rates of microtubule assembly, which were sufficiently high to account for the rates of microtubule polymerization observed *in vivo*, predicted disassembly rates that were simply too low to account for the rapid rates of depolymerization observed *in vivo*. This led Mitchison and Kirschner to examine the dynamics of individual microtubules *in vitro*. Tubulin heterodimer was diluted below the predicted critical concentration for microtubule polymerization. Surprisingly, some individual microtubules continued to grow under these conditions, behavior not predicted by polymerization equilibrium models. Mitchison and Kirschner provided a new model to explain these data, the Dynamic Instability Hypothesis (Mitchison and Kirschner, 1984; Mitchison and Kirschner, 1984).

The Dynamic Instability Hypothesis contended that microtubules existed at steady state rather than equilibrium. Within a population of microtubules, some individual microtubules grew, while others stochastically underwent catastrophic collapse. Kirschner and Mitchison predicted that the
Interconversion between growth and collapse was mediated by the presence of a GTP-tubulin "Cap" at the plus, or faster growing, end of the microtubules, which stabilized the microtubule ends. Loss of the GTP cap through tubulin GTP hydrolysis accounted for the rapid depolymerization of microtubules. They observed nucleator proteins at the minus, or slower growing, end of the microtubule polymer in vitro and predicted that microtubule polymerization from nucleators would be favored in vivo.

**Microtubule-associated Protein Hypothesis**

The third hypothesis that explains microtubule diversity is the Microtubule-associated Protein Hypothesis. The basic tenet of this hypothesis is that the innate property of tubulin heterodimers to polymerize is regulated and shaped by interactions between tubulin and other proteins. Interactions between these polypeptides and microtubules are responsible for the functional diversity of microtubules. One prediction of this hypothesis is that the expression of microtubule-associated proteins with specialized functions will be developmentally and spatially regulated.

This area of research is extremely active and numerous microtubule-associated proteins (MAPs) have been identified (Reviewed in: (Solomon, 1991; Solomon, et al., 1994)). MAPs can be categorized in one of several ways. First, associated proteins act as nucleators or microtubule organizing centers. Second, associated proteins are involved in interactions between microtubules and DNA at kinetochores. Third, associated-proteins known as motor proteins appear to mediate microtubule-based transport by traveling along microtubule polymers. Fourth, associated proteins are involved in the stabilization of microtubule polymers. Additional categories are also possible.

Unfortunately, an extensive discussion about the identification and characterization of MAPs and their roles in microtubule dynamics is beyond the scope of this dissertation. Since the research described in subsequent chapters has relevance to tubulin genes, biochemistry, and structure, the remainder of this chapter will focus on the literature related to these issues.
Introduction

Structural and Biochemical Properties of Tubulin

The Tubulin Genes

Alpha- and Beta-Tubulin Isoforms

Microtubules are primarily composed of two basic subunits, alpha- and beta-tubulin. They are highly homologous, with little sequence divergence between species. Extensive sequencing of alpha- and beta-tubulin genes in variety of organisms has revealed that most organisms contain multiple isoforms of alpha- and beta-tubulin. Chickens, for example, contain 5 alpha-tubulin genes and 5 beta-tubulin genes. In *Drosophila melanogaster*, there are 4 alpha- and 4 beta-tubulin isoforms (Little and Seehaus, 1988; MacRae and Langdon, 1989; Raff, et al., 1987).

Isoforms of alpha- and beta-tubulin have been categorized based on their characteristic divergent regions. Tubulin isoforms appear to be spatially and temporally regulated and beta-tubulin isoforms of the same class tend to be expressed in the same tissues of different organisms, lending support to the idea that they have evolved divergent functions. However, alpha-tubulin isoforms are not localized neatly according to class categories, revealing categorization or expression patterns may be complex (MacRae and Langdon, 1989). These data, taken together, appeared to support the Multi-tubulin Hypothesis because they suggested that at least some isoforms evolved because of specific cellular microtubule requirements.

A careful examination of the functional restrictions of tubulin isoforms have contradicted this major tenet of the Multi-Tubulin Hypothesis. First, in *S. cerevisiae*, where genetics is readily accessible, deletions in one of two alpha-tubulin isoforms are rescued by increased expression of the other isoform, demonstrating the isoforms are functionally interchangeable (Schatz, et al., 1986). In *A. nidulans*, divergent beta-tubulin isoforms are also functionally interchangeable (May, 1989). Second, the microtubules of the marginal band, a specialized microtubule structure characteristic of chicken erythrocytes, can be removed by detergent extraction, forming erythrocyte ghosts. The marginal band can be reconstructed in detergent-extracted cells by adding back calf brain microtubule preparations (Swan and Solomon, 1984). This experiment reveals that all the necessary structural information to form the marginal band...
must be contained in the erythrocyte ghosts, and conserved in calf brain tubulin. Third, chicken and yeast beta-tubulin chimeras containing a highly divergent carboxy-terminus from yeast beta-tubulin are stably expressed in tissue culture cells. The expressed chimeric beta-tubulin was incorporated into all cellular microtubules (Bond, et al., 1986). These data, taken together, strongly support the notion that even highly divergent tubulin isoforms are functionally interchangeable.

Results of experiments in Drosophila melanogaster demonstrated that some tubulin isoforms might have functional restrictions. D. melanogaster has a beta-tubulin isotype, B2t, expressed exclusively in testes (Kemphues, et al., 1979). The sequence of a second beta-tubulin isoform with developmentally regulated expression, B3, is highly divergent (Rudolph, et al., 1987). To test whether the B3 isoform was functionally equivalent to the B2t isoform, Elizabeth Raff's laboratory produced a construct containing B2t 5' non-coding sequence and B3 coding sequence and introduced it into cells. The recombinant protein accumulated to only 15% of the level of wildtype B2t and exhibited no detectable change in protein half-life over nine hours. In testes cells homozygous for B2t null and the construct, all microtubule structures were affected, although the cytoplasmic microtubules associated with mitochondrial derivatives were nearly normal. Axonemal structures were dramatically altered in strains containing the construct. These data demonstrated that the B2/B3 construct did not produce beta-tubulin capable of sustaining wildtype testicular microtubule structures (Hoyle and Raff, 1990).

These data did not show, however, that the two isoforms were not functionally interchangeable. The fact that all microtubule structures in B2/B3 strains were altered from wildtype strains showed that the expressed B3 sequence was insufficient to sustain any normal microtubule structure, suggesting the protein was defective in some way. That mitochondrial derivative microtubules were altered only slightly may be more a reflection of the sensitivity of these structures to tubulin alterations, rather than the ability of B3 to substitute for B2. Given that a number of mutations in B2t affect the axonemal complex (Fuller, et al., 1987; Kemphues, et al., 1982; Kemphues, et al., 1979), this structure may be particularly susceptible to tubulin alterations. That the proteins in B2/B3 strains only accumulated to 15% of wildtype levels
lends credence to the possibility that the expressed B3 is altered in some way. Negative results in experiments testing whether two isoforms are functionally interchangeable are extremely difficult to interpret because failure to function may occur for a number of reasons.

**Mutation analysis**

One prediction of the Multi-Tubulin Hypothesis is that conserved residues within tubulin genes are responsible for conserved function while divergent regions are involved specialized functions. The molecular and genetic tools available in *S. cerevisiae* and *D. melanogaster* have enabled researchers to examine the functional implications of mutations at specific amino-acids and regions of the alpha- and beta-tubulin genes in great detail.

A test of one element of the Multi-tubulin Hypothesis, that variable domains are necessary for species or cell-specific functions, was tested in *S. cerevisiae*. Constructs containing insertions of as many as 46 nucleotides (18 amino acids) were introduced into a variable domain near the amino-terminus of alpha-tubulin. Alpha-tubulin generated from these constructs, which provided the only source of alpha-tubulin in these cells, produced no phenotype (Schatz, *et al.*, 1987).

The carboxy-terminus of beta-tubulin represents a second variable domain, used in the classification of beta-tubulins. The beta-tubulin of *S. cerevisiae* contains twelve amino-acids at the carboxy-terminus not found in other sequenced beta-tubulins. Deletion of this sequence, resulted in no phenotype other than a slight increase in sensitivity to the anti-mitotic drug benomyl (Katz and Solomon, 1988). As the number of amino-acids deleted from the carboxy-terminus of beta-tubulin increased, the phenotypes became increasingly severe (Matsuzaki, *et al.*, 1988), showing that alterations in conserved rather than divergent regions had serious repercussions. In *D. melanogaster*, beta-tubulin generated from genes in which early stop codons at 276 or 397 were introduced, did not accumulate. Beta-tubulin containing a deletion of 15 amino-acids at the carboxy-terminus produced less stable protein than the wildtype gene, but this beta-tubulin supported nearly normal microtubule structures. The exceptions was the axonemal complex, which is likely particularly susceptible to altered protein stability (Fackenthal, *et al.*, 1993; Kemphues, *et al.*, 1982).
Expression of a chicken:yeast beta-tubulin chimera in tissue culture cells produced similar results. Beta-tubulin produced from constructs containing 25% yeast sequence at the carboxy-terminus assembled into all microtubules. As the proportion of yeast sequence increased, beta-tubulin was less stable and assembled at lower efficiency than wildtype protein (Fridovich-Keil, et al., 1987). These data show that cells were less sensitive to changes in the variable carboxy-terminal domain of beta-tubulin than to changes in conserved domains, contradicting one tenet of the Multi-tubulin Hypothesis.

To examine whether small regions in the primary sequences of alpha- and beta-tubulin were responsible for specific microtubule functions, a number of mutations were generated in the tubulin genes of *S. cerevisiae*, *D. melanogaster*, and other organisms (Kemphues, et al., 1982; Reijo, et al., 1994; Schatz, et al., 1988; Sullivan and Huffaker, 1992; Thomas, et al., 1985). A number of these mutations, such as those affecting GTP hydrolysis, will be discussed below.

One mutation, a glutamic acid to lysine alteration at position 288 of beta-tubulin exhibited a particularly intriguing structural phenotype. Microscopic examination of cross-sections of microtubules from Bt2^8^ mutant strains were S- or U-shaped rather than O-shaped. Since the mutation occurred in an almost absolutely conserved residue, these data suggested a specific defect in heterodimer interaction within the microtubule polymer. In Chapter Three of this dissertation, I describe the consequences of introducing an identical mutation into the sole beta-tubulin of *S. cerevisiae*.

**Regulation of Tubulin mRNA**

One of the predictions of the Dynamic Instability Hypothesis is that microtubule structures are sensitive to changes in the availability of the soluble tubulin heterodimer. While there are numerous means by which a cell might regulate the availability of tubulin heterodimer, such as sequestration of tubulin heterodimers or modification of heterodimers to assembly-incompetent forms, one mechanism, the regulation of alpha- and beta-tubulin protein levels by the regulation of tubulin messenger RNA levels, was examined extensively in a series of experiments.
Introduction

The identification of tubulin mRNA (Cleveland, et al., 1978) enabled researchers to examine the sensitivity of tubulin message and protein in response to microtubule-depolymerizing and stabilizing drugs. $^{35}$S-Methionine protein pulse label experiments and quantitative northern blotting analysis revealed that tubulin message levels and the rate of tubulin synthesis decreased after the addition of colchicine and nocodazole to cellular growth media, drugs that decreased the level of tubulin in polymeric form. Addition of taxol or vinblastine, drugs which increased the level of polymeric tubulin or decreased soluble tubulin, did not cause decreased tubulin message levels or protein synthesis. These authors concluded that tubulin message levels were sensitive to changes in the pool of tubulin heterodimer (Ben-Ze'ev, et al., 1979; Cleveland, et al., 1981).

Tubulin microinjected into cells also induced "autoregulation" of tubulin message and new tubulin synthesis. Oddly, lower concentrations of purified tubulin, of which only a fraction was assembly competent, decreased mRNA levels more efficiently than thrice-cycled tubulin preparations or colchicine-bound tubulin (Cleveland, 1983). These data suggested that the regulation of tubulin message might be a response to partially degraded or denatured tubulin rather than to changes in the tubulin soluble pool.

Tubulin autoregulation appeared to occur in the cytoplasm, because enucleated cells treated with colchicine or colcemid, a microtubule depolymerizing drug similar to colchicine, regulated tubulin message levels (Caron, et al., 1985b; Pittenger and Cleveland, 1985). Colchicine-induced autoregulation occurred in the presence of cycloheximide, a drug which inhibits translation, but does not inhibit translation initiation. Cells treated with puromycin and pactamycin, which cause premature release of transcripts or inhibit translation initiation, respectively, did not regulate tubulin message levels (Pachter, et al., 1987). Taken together, these data point strongly to a mechanism of message regulation and degradation in the cytoplasm that requires translational initiation.

The first four amino acids of the beta-tubulin sequence, MR(E/D)I, were sufficient for colchicine-induced autoregulation to occur, but constructs containing the tetra-peptide sequence did not autoregulate unless they were sufficiently long to code for at least 41 additional amino-acids. (Gay, et al., 1987;
Yen, et al., 1988a; Yen, et al., 1988b). Since the fourth amino-acid is not conserved between alpha- and beta-tubulin, the requirement for isoleucine was carefully examined. Constructs containing isoleucine exhibited message level autoregulation, while polar residues at position four were only mildly autoregulatory. Cysteine, located at position four of alpha-tubulin, did not confer autoregulatory properties to the constructs tested. In fact, of several transcripts containing alpha-tubulin sequence introduced into cells, including one which contained the MREI sequence, none was autoregulated (Bachurski, et al., 1994). These data implied that alpha- and beta-tubulin mRNA were not regulated in the same manner. It seems rather remarkable that two separate mechanisms evolved for regulating the same phenomena for interacting proteins.

There are a number of additional concerns about tubulin message autoregulation. First, tubulin protein half-life is extremely long -- 50 hours in some cases (Caron, et al., 1985b). It is unlikely that a mechanism would evolve to regulate the level of a long-lived protein by regulating the level of messenger RNA for that protein. A more likely mechanism, alterations in protein stability, has been demonstrated to occur in vivo (Gong and Brandhorst, 1988). Newly synthesized tubulin may be particularly susceptible to degradation. In cultured neuronal cells treated with LiCl, newly synthesized tubulin was preferentially degraded (Bennett, et al., 1991). Second, cells plated on low concentrations of laminin decreased microtubule length and increased the soluble tubulin pool. New tubulin synthesis decreased in these cells, consistent with the tubulin autoregulatory hypothesis. However, the tubulin half-life increased, consistent with thermodynamic models that suggested higher concentrations of heterodimer were required to sustain microtubules in round cells (Mooney, et al., 1994). Third, researchers were unable to detect a physical interaction between tubulin heterodimer and the amino-terminal peptide required for tubulin message level regulation (Theodorakis and Cleveland, 1992). Fourth, the precise consequences of microtubule-depolymerizing drugs were unknown, making it difficult to ascertain the specific event triggering the autoregulatory response. Fifth, abrupt alterations in polymer stability were unlikely to occur in normal cells, making it a concern that the autoregulatory response may not be a physiologically relevant event. Sixth, S. cerevisiae cells with 2 copies of the
Introduction

major alpha-tubulin gene to one copy of beta-tubulin decreased the level of alpha-tubulin message and protein to the match that of beta-tubulin, to near 50% of wildtype levels (Katz, et al., 1990). These cells were phenotypically normal, and there were no adverse affects to dramatic alterations in tubulin protein levels. These results suggest it is unlikely that tubulin message autoregulation is the primary mechanism involved in regulating tubulin heterodimer availability, an important component of the Dynamic Instability Hypothesis.

Tubulin Protein Folding

The correct three-dimensional structure of alpha- and beta-tubulin proteins are critical for the function of these polypeptides. While some proteins appear to contain sufficient information in their primary sequences to specify correct protein folding and secondary structure, for tubulin folding is mediated by chaperonins. Chaperonins are a family of proteins, often toroidal-shaped complexes made up of several subunits. Discovery of the role chaperonins play in correctly folding alpha- and beta-tubulin occurred in experiments characterizing the protein products of the \textit{in vitro} translation of tubulin genes.

Attempts to produce alpha- and beta-tubulin in \textit{E. coli} in order to examine the biochemical and physical properties of these proteins were unsuccessful because the proteins aggregated (Gao, et al., 1993; Zabala and Cowan, 1992). When tubulin message was translated \textit{in vitro} in rabbit reticulocyte lysate preparations, 67% of the alpha-tubulin but only 13% of the beta-tubulin was competent to assemble into microtubules (Cleveland, et al., 1978). Subsequent experiments have characterized the protein products of \textit{in vitro} translation systems more carefully.

Alpha-tubulin eluted as a single peak from anion exchange columns and appeared to be monomeric based on its mobility. Co-immunoprecipitation experiments failed to identify interactions with beta-tubulin from the rabbit reticulocyte lysate preparations. The alpha-tubulin produced \textit{in vitro} interacted and cycled with purified brain microtubules (Yaffe, et al., 1988b). However, examination of alpha-tubulin mobility during native polyacrylamide gel electrophoresis revealed the protein was part of a 900 kDa complex (Zabala and Cowan, 1992).
Three forms of beta-tubulin eluted from anion exchange columns, as 55 kDa (BII), 110 kDa (BIII), and 900 kDa (BI) proteins (Yaffe, et al., 1988a), with consistent results in native gel electrophoresis assays (Zabala and Cowan, 1992). Nearly all of the BIII dimeric fraction, composed of both rabbit alpha-tubulin and chicken beta-tubulin, cycled with purified brain microtubules, but only 20-35% of the BII monomeric fraction cycled with exogenous microtubules. A portion of the tubulin from the BI and BIII fractions ran as 110 kDa protein after exposure to exogenous microtubules, and contained alpha-tubulin, as assayed by co-immunoprecipitation with anti-alpha-tubulin antibodies (Yaffe, et al., 1988a). Addition of purified brain microtubules and GTP produced more beta-tubulin in the dimeric fraction (Yaffe, et al., 1992; Zabala and Cowan, 1992). Pulse chase analysis revealed that labeled tubulin moved from BI to BII in the absence of exogenous tubulin and to BIII with exogenous microtubules. These data suggested the BI form is an intermediary between newly synthesized beta-tubulin and tubulin heterodimer (Yaffe, et al., 1992).

One of the proteins present in the tubulin 900 kDa complex was TCP1 (Frydman, et al., 1992), a chaperonin-like protein with some sequence homology to GroEL, but structurally and biochemically unique (Frydman, et al., 1992). TCP1 formed a complex with seven or eight other proteins from rabbit reticulocyte lysates and bovine testes, as assayed by denaturing gel electrophoresis (Rommelaere, et al., 1993).

The interaction between tubulin and the chaperonin protein TCP1 occurred in a 900 kDa complex in vivo in CHO cells, with labeled tubulin moving from the BI to BIII fractions in pulse chase experiments (Sternlicht, et al., 1993). In vitro folding assays with purified TCP1 protein complex proteins from bovine testes demonstrated chaperonin-mediated folding of tubulin into functional heterodimers in the presence of GTP and exogenous microtubules (Frydman, et al., 1992; Rommelaere, et al., 1993). Actin and gamma-tubulin also interacted with TCP1 complex homologs (Gao, et al., 1992; Melki and Cowan, 1994; Melki, et al., 1993).

Characterization of proteins in rabbit reticulocyte lysate preparations identified two protein cofactors, A and B, required, along with exogenous microtubules, to release alpha- and beta-tubulin from chaperonin complexes, in vitro. The resultant alpha- and beta-tubulin polypeptides were competent to co-
assemble with microtubules. Cofactor A alone released monomeric beta-tubulin, but it was not assembly-competent (Gao, et al., 1993). Cofactor A was purified based on its ability to release beta-tubulin. The protein was 28 kDa, expressed abundantly in testis, and had no similarity to known proteins (Gao, et al., 1994).

A S. cerevisiae TCP1 homolog was identified in a screen for genes with homology to mouse and Drosophila TCP1. The gene was essential. Temperature sensitive alleles produced excess unbudded anucleated or multinucleated cells. The strains were sensitive to the anti-mitotic drug benomyl and exhibited altered alpha-tubulin immunofluorescence staining (Ursic and Culbertson, 1991). A second member of the TCP1 family, TCP1beta, was identified based on its homology to TCP1alpha. This gene was also essential. Additional proteins co-immunoprecipitated with antibodies to TCP1alpha under non-denaturing conditions, suggesting the yeast TCP1 homologs resided in a protein complex (Miklos, et al., 1994). TCP1beta (named BIN3) and a third member of the chaperonin/TCP1 family (BIN2) were identified as suppressors of strains with excess binucleated cells. Mutations in BIN2 and BIN3 exhibited defective microtubule and actin phenotypes. Double mutants were inviable (Chen, et al., 1994). ANC2, a fourth member of the TCP1 family, was identified as a suppressor of defects that enhance actin mutations. It was an essential gene. Temperature sensitive mutants exhibited cellular phenotypes similar to those produced with actin mutants; they had delocalized actin structures. ANC2 mutant strains also exhibited a slight microtubule defect and were sensitive to benomyl (Vinh and Drubin, 1994). The phenotypic consequences of chaperonin homolog mutants in yeast demonstrate a critical role for these proteins, and suggest a direct interaction between TCP1 and tubulin and/or actin. However, the microtubule- or actin-related phenotypes could be due to indirect or downstream consequences of defects in these genes.

Structure and Biochemistry of Tubulin

Tubulin Protein Structure

The primary sequences of alpha- and beta-tubulin have been extensively characterized, but the secondary structure of the alpha- and beta-tubulin
polypeptides is still poorly understood. Research in this area has been limited by the technical difficulties in producing native alpha- or beta-tubulin polypeptides in sufficient quantities for biochemical and structural studies. As described above, \textit{in vitro} translation of tubulin genes produced only small quantities of monomeric alpha- and beta-tubulin and the ability of these proteins to assemble was uncertain. Attempts to separate purified tubulin heterodimer into functional monomeric alpha- and beta-tubulin have also been unsuccessful. Alpha- and beta-tubulin polypeptides from heterodimer pools were separable by gel electrophoresis techniques (Bryan and Wilson, 1971) and by HPLC (Stephens, 1988), but these techniques did not preserve native structure. Crystal structures of alpha- or beta-tubulin, which would provide extensive structural information about these polypeptides, have not been reported.

In the absence of techniques to produce large quantities of native monomeric alpha- and beta-tubulin, structural studies of the alpha- and beta-tubulin polypeptides have chiefly utilized pure sources of heterodimeric protein. Experiments examining tubulin monomeric and heterodimeric structure fall into several general classes: proteolysis accessibility, antibody epitope accessibility and obstruction; biophysical chemistry studies; and microscopy experiments. These experiments have provided information about the general structure of the tubulin heterodimer, although much remains to be elucidated.

Early experiments with proteases such as thermolysin, trypsin, and chymotrypsin demonstrated that unpolymerized alpha- and beta-tubulin were more accessible to proteases than polymerized alpha- and beta-tubulin (Brown and Erickson, 1983). These data suggested that proteolytic sites were differentially accessible due to the structural conformations of tubulin in polymerized and unpolymerized forms. Antibodies generated against gel-purified full-length alpha- or beta-tubulin made it possible to identify characteristic alpha- or beta-tubulin proteolytic fragments by size (Mandelkow, \textit{et al.}, 1985). Proteolysis of beta-tubulin, followed by covalent cross-linking, and separation by gel electrophoresis identified an interaction between the small 20 kDa beta-tubulin domain and a 55 kDa protein, presumably alpha-tubulin. The larger 30 kDa beta-tubulin domain did not cross-link. Similar experiments with alpha-tubulin demonstrated an interaction between a 55 kDa protein,
presumably beta-tubulin, and the large N-terminal domain of alpha-tubulin. These data led to a model of heterodimer interactions. Tubulin heterodimer is composed of four domains, two in alpha-tubulin and two in beta-tubulin. The small alpha-tubulin domain interacts with the large beta-tubulin domain, and the small beta-tubulin domain interacts with the large alpha-tubulin domain (Kirchner and Mandelkow, 1985).

Antibodies generated against unique peptides of alpha- and beta-tubulin, enabled researchers to extensively characterize tubulin cleavage patterns. Data from proteolysis experiments showed a cluster of proteolytic cleavages around 115-165, 260-300, and the carboxy-terminus of each tubulin chain, suggesting three tubulin domains, separated by protease accessible sections (de la Viña, et al., 1988). X-ray studies of microtubule polymers at 18Å resolution produced images of tubulin polymers with a repeated motif pattern in the polymer also suggestive of three domain repeats in the tubulin heterodimer. Unfortunately, the relative positions of alpha- and beta-tubulin could not be determined from this analysis (Beese, et al., 1987).

A second method used to characterize tubulin heterodimer structure relied on the hypothesis that antibodies would be able to stain microtubules only if the epitopes were present at the microtubule surface, allowing identification of these regions. Antibodies with epitopes at the carboxy-terminus of alpha- or beta-tubulin strongly stained microtubules, suggesting these domains resided at the microtubule surface (Breitling and Little, 1986; Draber, et al., 1989). In contrast, antibodies generated against amino-terminal domains of tubulin stained microtubules poorly, suggesting these domains reside within the microtubule polymer (Draher, et al., 1989).

Subsequent research has focused extensively on the carboxy-terminal domains of alpha- and beta-tubulin, revealing potential interaction sites between tubulin and the microtubule-associated proteins MAP2 and tau. In overlay experiments, iodinated MAP2 and tau associated with proteolytic fragments located in the carboxy-terminal regions of alpha- and beta-tubulin (Littauer, et al., 1986). Peptides generated from the alpha- and beta-tubulin sequences responsive to MAPs inhibited binding of tau and MAP2 to microtubule polymers (Maccioni, et al., 1988). Antibodies generated against the carboxy-terminal regions interfered with MAP-induced microtubule
polymerization, although these microtubules polymerized under conditions where MAPs were not essential (Rivas, et al., 1988; Vera, et al., 1988).

Classical biophysical chemistry tools, such as fluorescence spectra, circular dichroism, Raman spectroscopy and sedimentation equilibrium have also been used to examine properties of tubulin secondary structure. These techniques have been particularly useful in identifying the structural consequences of a number of buffer conditions, including pH, altered temperatures, incubation time, and the presence or absence of GTP/GDP, colchicine, proteases, etc. (see for examples, (Audenaert, et al., 1989; Kanazawa and Timasheff, 1989; Mozo-Villarias, et al., 1991; Vera, et al., 1989). Unfortunately, the resolution of these techniques in characterizing alpha- and beta-tubulin secondary structure is imprecise.

In Chapter 3 of this dissertation, I will describe a procedure for isolating tubulin protein preparations greatly enriched for alpha- and beta-tubulin monomer. This procedure, which produces alpha- or beta-tubulin functional by at least one criteria, GTP binding, may be useful for tubulin structural studies.

**The Tubulin Heterodimer**

Microtubules, originally purified based on their colchicine binding activity, were found to be composed of an active subunit of ~120 kDa, which could be converted into a ~60 kDa unit under denaturing conditions (Weisenberg, et al., 1968). The ~60 kDa component could be separated electrophoretically after exposure to reducing agents into two protein bands, with distinguishable amino-acid compositions. These data showed that microtubules were composed of heteropolymers. The authors hypothesized that the 1 to 1 stoichiometry was consistent with the presence of heterodimer subunits, although homo-dimers could not be ruled out (Bryan and Wilson, 1971). On native gels, purified microtubule protein exhibited a complex pattern, with protein present at ~55 kDa, ~110 kDa, ~220 kDa, ~330 kDa, etc. Denaturation by exposure to 8M urea reduced the pattern to two protein bands, each sized ~55 kDa (Lee, et al., 1973). These data suggested that microtubules were composed of two, non-identical proteins, present in ~1 to 1 stoichiometry, that formed a ~110 kDa subunit. These experiments could not distinguish whether
the basic subunits of microtubules were composed of equal quantities of alpha/alpha and beta/beta subunits, or exclusively alpha/beta subunits.

The evidence that microtubules were composed of tubulin heterodimer was derived from several indirect experiments. First, Luduena and colleagues (Luduena, et al., 1977) cross-linked tubulin protein and examined the products under gel electrophoresis to distinguish alpha-alpha, beta-beta, and alpha-beta dimeric forms. The alpha-beta forms were as abundant as alpha-alpha forms, and slightly more abundant than beta-beta forms. The researchers hypothesized that if the interactions between alpha-alpha and beta-beta were non-specific, their frequency should increase under conditions where aggregation increased, such as long incubation times. The opposite result should occur if alpha-beta interactions were non-specific. They found increased beta-beta forms as incubation time increased. When microtubules were incubated with vinblastine and colchicine, conditions where denaturation and aggregation would be inhibited, the dominant cross-linked species was alpha/beta, comprising greater than 90% of the cross-linked species when both drugs were present. Unfortunately, cross-linking efficiency in these experiments was poor; only 10% of the protein was cross-linked, of which 7.6% was dimeric, with the remaining 2.4% aggregated (Luduena, et al., 1977). As a result, only a small fraction of tubulin protein was actually characterized. Protein preparations highly enriched for alpha- or beta-tubulin show formation of alpha-alpha or beta-beta homodimers, based on migration and alpha- and beta-tubulin localization on native gels western blots (this thesis, see Appendix One), demonstrating these forms occur under some conditions.

A second line of evidence that the basic microtubule subunit is composed of alpha- and beta-tubulin heterodimers is based on results from antibody experiments. In microtubule structures, alpha- and beta-tubulin consistently co-localized, based on indirect immunofluorescence experiments using antibodies specific for alpha- or beta-tubulin. Alpha- and beta-tubulin co-immunoprecipitated with antibodies generated against either tubulin chain, demonstrating these proteins directly interacted with one another (For example, Chapter Three, Figure 3.1).

Third, the results of genetic experiments in S. cerevisiae have provided evidence for a direct interaction between alpha- and beta-tubulin. Screens for
suppressors of mutations in alpha-tubulin identified beta-tubulin and screens for suppressors of mutations in beta-tubulin identified alpha-tubulin (Stearns and Botstein, 1988). These data showed that alpha- and beta-tubulin participated in the same cellular structure, and suggested a direct interaction.

**Heterodimer Dissociation**

There have been a series of biochemical experiments measuring the strength of the association between alpha- and beta-tubulin. The experiments fall into three general categories: sedimentation equilibrium experiments, change in proteolysis susceptibility, and the change in the fluorescence pattern of labeled tubulin. These techniques have produced a range of heterodimer dissociation constants, between \( K_D = 1 \times 10^{-7} \) M and \( 1 \times 10^{-6} \) M. While some of the variability in the tubulin heterodimer dissociation constant values was certainly due to differences in experimental approach, some was also due to differences in temperature, pH, and buffer conditions at which the experiments were performed.

The earliest measurements of tubulin heterodimer dissociation used sedimentation coefficient equilibrium techniques. Within a sedimentation pattern, the tubulin heterodimer was present in a molecular weight distribution between 80,000 to 110,000 kDa, with the average dependent upon the starting concentration of tubulin. The molecular weights represented a continuum of rapidly equilibrating monomer and dimer. From these data, the authors extrapolated a dissociation constant for tubulin dimer at \( K_D = 8 \times 10^{-7} \) M at 4 °C in Pipes buffer (pH = 6.9, with EGTA, MgSO\(_4\), GTP, and DTE). Unfortunately, this technique required 15 to 30 hours. Although 80% of the tubulin maintained at 4 °C in this buffer retained the ability to polymerize, it is difficult to know the effects of prolonged incubation on the dissociation constant (Detrich III and Jr., 1978). Shorter columns and faster equilibration times yielded a slightly stronger association, \( K_D = 2 \times 10^{-7} \) M at 4 °C in MES buffer (pH = 6.9, with MgCl\(_2\), EGTA, and GTP) (Sackett and Lippoldt, 1991). In the absence of guanine nucleotides, the association between alpha- and beta tubulin was weakened slightly, with \( K_D = 1 \times 10^{-6} \) M at 10 °C in sodium phosphate buffer (with EGTA), as measured by sedimentation equilibrium (Shearwin, *et al.*, 1994a).
A second method to measure the dissociation constant of tubulin heterodimer took advantage of the different susceptibilities of tubulin monomer and dimer to subtilisin protease activity. By treating decreasing concentrations of tubulin heterodimer with subtilisin and measuring the resultant proteolytic activity, the authors could estimate a dissociation concentration. In these experiments, the measured tubulin heterodimer dissociation constant was $K_D = 1.7 \times 10^{-7}$ M in MES buffer, (with $\text{MgCl}_2$, EGTA, and GTP). It was difficult, however, to ascertain whether the dissociation reached equilibrium in the 30 to 45 minute experiments (Sackett and Wolff, 1986; Sackett, et al., 1989).

The third method to measure tubulin heterodimer dissociation constants relied on the change in fluorescence pattern for labeled tubulin heterodimer. The change of fluorescence of Nile Red, a probe for non-polar surfaces of proteins, was used as a probe of tubulin heterodimer dissociation, yielding a $K_D = 1 \times 10^{-6}$ M in MES buffer, ($\text{MgCl}_2$, EGTA, and GTP) (Sackett, et al., 1990). Assembly-competent tubulin labeled with DTAF or FITC was examined at different concentrations to measure the anisotropy of fluorescence. These experiments yielded a dissociation constant of $8.4 \times 10^{-7}$ M at 20 °C in Pipes buffer (with EGTA and $\text{MgSO}_4$) (Mejillano and Himes, 1989) or $7.2 \times 10^{-7}$ M in Pipes buffer (EGTA, $\text{MgSO}_4$, and GTP) (Panda, et al., 1992). Increased temperature, from 20°C to 36°C, increased the measured $K_D$ two-fold and decreased temperature, to 10°C, decreased it two-fold (Mejillano and Himes, 1989).

**Buffer Components**

The strength and stability of the association between alpha- and beta-tubulin and the tubulin polymerization reaction are sensitive to buffer conditions. Numerous buffer components have been examined to determine their effects on the tubulin heterodimer. While some of these components affect the dissociation constant of the tubulin heterodimer, others primarily affect microtubule assembly or disassembly.

**Glycerol and Sucrose**

Glycerol and sucrose promote both the stability of the tubulin heterodimer and microtubule assembly. The earliest purification of tubulin heterodimer, based on its interaction with colchicine, included sucrose in the purification.

**Detergent**

The physical interactions between tubulin and a variety of detergents, such as Triton X-100, octylglucoside, and deoxycholate, have been extensively studied. Deoxycholate and octylglucoside had moderate effects on tubulin colchicine binding activity while Triton X-100 had very little effect, retaining 88% of the binding activity of tubulin without detergent. Nearly total binding activity was restored upon removal of the octylglucoside and deoxycholate detergents (Andreu, 1982) showing that short term exposure to detergents did not have permanent effects on tubulin colchicine binding. Deoxycholate and octylglucoside produced moderate changes in the UV circular dichroism and fluorescence patterns of tubulin, qualitatively different from changes induced by sodium dodecylsulfate (SDS) denaturation. The patterns suggested slight unfolding of the tubulin heterodimer (Andreu, 1982; Andreu, 1986a; Andreu and Munoz, 1986b; Andreu, et al., 1986c). Tubulin with or without deoxycholate was analyzed by X-ray scattering using synchrotron radiation. The results demonstrated a detergent-mediated swelling on the protein (Andreu, et al., 1989). Tubulin was more susceptible to proteases in detergent buffers (Andreu and Munoz, 1986b), consistent with the idea that detergents alter protein structure.

The temperature dependence of heterodimer dissociation constants measured by sedimentation equilibrium suggested heterodimers are held together by hydrophobic forces (Sackett and Lippoldt, 1991), as did
measurements using a second method, alterations in Nile Red fluorescence (Sackett, et al., 1990). Decay of tubulin colchicine binding activity was accompanied by an increase in binding sites for Bis(8-anilinonaphtalene-1-sulfonate), indicating the appearance of hydrophobic regions as tubulin denatured (Prasad, et al., 1986). Detergents may interact with the hydrophobic alpha/beta interface, destabilizing the heterodimer and making the protein incompetent to assemble. Octylglucoside and deoxycholate inhibited the self-assembly of tubulin into microtubules, but the effect was reversible (Andreu, 1982). A milder detergent, Triton X-100, did not interfere with tubulin self-assembly (Friden, et al., 1987), demonstrating it had slight effects on tubulin hydrophobic interactions.

In chapter three of this dissertation, I will discuss the in vitro separation of alpha- and beta-tubulin from heterodimer using Triton X-114 or Nonidet P-40. While it is not clear why the detergent-mediate separation of alpha- and beta-tubulin occurs, work from tubulin heterodimer dissociation and detergent interaction experiments suggest the detergent interferes with heterodimer interactions, favorably altering the dissociation constant.

**Other buffer components and conditions**

A number of other buffer components and conditions have been examined, including drugs which affect the microtubule heterodimer or polymer state, buffer type, buffer pH, buffer temperature, and salt concentration. An extensive review of all the literature related to these topics is not possible, so I will briefly describe a few experiments examining these parameters.

The polymerization of purified microtubules was not sensitive to buffer type. Studies demonstrated no significant change in tubulin heterodimer folding properties, as assayed by circular dichroism, or tubulin polymerization in a number of commercially available buffers, such as Tris, MES, or Pipes (Lee and Timasheff, 1977). Altering buffer pH from 6.0 to 7.0, however, resulted in increased polymerization (Lee and Timasheff, 1977). Buffer pH greater than 9.0 resulted in irreversible tubulin denaturation. Tubulin in pH 8.0 buffer was competent to assemble only if the buffer contained either 2 M glycerol or 1 mM GTP (Brown-Croom, et al., 1986). High salt concentrations, > 0.7 M NaCl caused irreversible aggregation of tubulin heterodimer (Brown-Croom, et al., 1986).
A number of interactions between microtubules and anti-mitotic drugs, such as colchicine, benomyl, and taxol, have been experimentally examined. The best characterized is colchicine, which depolymerizes microtubules. The interaction is direct, as tubulin co-purifies with colchicine in treated cells with one mole of colchicine per mole of heterodimer (Weisenberg, et al., 1968). Colchicine stabilizes the tubulin heterodimer, since it increased the association constant for tubulin heterodimer (Detrich III, et al., 1982; Sackett and Lippoldt, 1991). Colchicine may preferentially interact with beta-tubulin. In cross-linking experiments, colchicine was cross-linked to beta-tubulin (Wolff, et al., 1991) and beta-tubulin, but not alpha-tubulin, was more sensitive to proteases when colchicine was present (Sackett and Varma, 1993).

Taxol, isolated as an anti-tumor agent purified from the Pacific Yew tree, had the unique property of stabilizing microtubule polymers, making them resistant to temperature- or calcium-induced depolymerization (Schiff, et al., 1979). Purified tubulin assembled without GTP or MAPs in the presence of taxol (Collins and Valee, 1987; Diaz and Andreu, 1993; Schiff and Horwitz, 1981) and taxol-treated cells exhibited bundles of microtubules, were cold-resistant, and did not migrate properly (Schiff and Horwitz, 1980). 0.6 mol of labeled taxol bound to 1 mol of tubulin in vitro, with a $K_{\text{app}}$ of $8.1 \times 10^{-7}$ M for taxol to tubulin monomer (Parness and Horwitz, 1981). Although the precise action of taxol is unknown, taxol likely binds along the length of the microtubules, halting microtubule disassembly when the taxol molecule is encountered (Caplow, et al., 1994).

Benomyl is an anti-mitotic drug used in a number of organisms, including S. cerevisiae. Methyl Benzimidazol-2-YL Carbamate (MBC), the active breakdown product of benomyl, directly bound to tubulin. The binding was competitively inhibited by colchicine and vinblastine, suggesting the drug may act at the same site as those microtubule dissolution drugs (Davidse and Flach, 1977). In S. cerevisiae, treatment with MBC produced large-budded cells and spindle-pole duplication but not separation (Quinlan, et al., 1980). The precise action of the drug is uncertain.
**Interactions with Small Molecules**

**Calcium**

Tubulin is polymerized and depolymerized by warm or cold, respectively. Ca\(^{++}\) in the assembly buffer mimicked the affects of cold-induced depolymerization of microtubules (Weisenberg, 1972). In sedimentation experiments, tubulin depolymerized by exposure to Ca\(^{++}\) exhibited the same properties as tubulin depolymerized by exposure to cold (Kirschner, *et al.*, 1974). Two different types of Ca\(^{++}\)-binding sites on tubulin were identified. The high affinity site, \(K_D = 3.2 \times 10^{-6}\) M, bound one mol of Ca\(^{++}\) to one mol of tubulin. There were 16 low affinity sites, with \(K_D = 2.5 \times 10^{-4}\) M (Solomon, 1977). Subtilisin digestion of the tubulin molecule, which cleaves the carboxy-terminus of alpha- and beta-tubulin (S-tubulin) resulted in the loss of the high affinity Ca\(^{++}\)-binding site. S-tubulin polymerized at 0.5 mM Ca\(^{++}\), a concentration that inhibited polymerization of tubulin under the same conditions (Serrano, *et al.*, 1986). These results suggested that Ca\(^{++}\) was associated with both alpha- and beta-tubulin subunits at the carboxy-terminus. Alternatively, subtilisin cleavage of tubulin altered tubulin structure such that binding at the high affinity Ca\(^{++}\)-binding site was inhibited.

**Guanine Nucleotides and Mg\(^{++}\)**

*GTP-Tubulin Dissociation Constants*

One of the earliest interactions between tubulin and small molecules to be identified was the interaction between tubulin and GTP. When the tubulin heterodimer was first purified, based on its interactions with the microtubule-depolymerizing drug colchicine, two moles of GTP were associated with each mole of tubulin heterodimer. Approximately half of the GTP was exchanged (Weisenberg, *et al.*, 1968) within 5 minutes (Arai and Kaziro, 1977). The second mol of GTP remained stably-associated with tubulin in these experiments, demonstrating the presence of two different GTP binding sites in the tubulin heterodimer. The interactions between tubulin and guanine nucleotides have subsequently been examined by a number of laboratories in great detail.
The GTP-tubulin dissociation constant at the exchangeable site (E-site), measured in several laboratories, was $K_D = 0.5 \times 10^{-6}$ M to $2.2 \times 10^{-8}$ M, depending on the particular experimental approach and buffer condition. Measurements of the dissociation constant for interactions between tubulin and GDP, which competes with GTP for binding at the E-site, ranged between $K_D = 1.9 \times 10^{-6}$ M and $K_D = 8.3 \times 10^{-8}$ M (Arai and Kaziro, 1977; Correia, et al., 1987; Fishback and Yarbrough, 1984; Zeeberg and Caplow, 1979). A number of buffer components had dramatic consequences for the relative affinities of tubulin and guanine nucleotides.

The Effects of Mg++ on Guanine Nucleotide Binding

The presence of Mg++ in experimental buffer systems dramatically increased the affinity between GTP and the tubulin E-site. Comparative association constants for tubulin-GTP with or without Mg++ were $K_a = 6.4 - 9.0 \times 10^7$ M$^{-1}$ and $K_a = 4.0 \times 10^4$ M$^{-1}$, respectively. In contrast, the tubulin-GDP association constant did not change dramatically with or without Mg++ in the experimental buffer, $K_a = 2.5 - 2.7 \times 10^7$ M$^{-1}$ and $K_a = 1.6 \times 10^7$ M$^{-1}$, respectively (Correia, et al., 1987). Similar results on the effect of Mg++ on GTP-tubulin binding constants have been obtained in other laboratories (Grover and Hamel, 1994; Mejillano and Himes, 1991). Apparently, GTP competes favorably with GDP for binding at the E-site when Mg++ is present and poorly in the absence of Mg++. The physiological relevance of these data are unclear, since dramatic alterations in Mg++ concentrations would affect a number of cellular systems.

The GTP Nonexchangeable Site

The second site of GTP binding to tubulin, the non-exchangeable site (N-site), was characterized by Spiegelman, Penningroth, and Kirschner. Chinese hamster ovary tissue culture cells, grown in the presence of $^{32}$P, were cycled in the presence of unlabeled GTP to replace the GTP at the exchangeable binding site on tubulin. Chromatography resolved the nonexchangeable $^{32}$P as GTP, not GDP or GMP. Approximately 0.7 to 1.1 mol of $^{32}$P-GTP were bound per mole of tubulin. The half-life of GTP at the N-site, determined from pulse chase experiments, was 15 hours in stationary cells and 33 hours in growing cells. The tubulin protein half life in stationary and growing cells was 48 hours and 34 hours, respectively (Spiegelman, et al., 1977). Mg++ was found to interact with guanine nucleotides at the tubulin N-site. Unlike the association between Mg++
and guanine nucleotides at the E-site, Mg++ interacted exclusively with GTP at the N-site (Correia, et al., 1987).

*The Effects of Guanine Nucleotides on Heterodimer Stability*

The role guanine nucleotide binding plays with respect to heterodimer intramolecular interactions has been extensively studied. The tubulin heterodimer was stabilized by the presence of guanine nucleotides in the experimental buffer system, based on sedimentation equilibrium dissociation constant experiments, with GDP/Mg++ more effectively stabilizing the tubulin heterodimer than GTP/Mg++ (Sackett and Lippoldt, 1991; Shearwin, et al., 1994a). GTP/Mg++ protected the tubulin heterodimer against denaturation (Arai and Kaziro, 1977) and chymotrypsin-induced changes in the heterodimer secondary structure (Arai and Kaziro, 1977). The presence of GTP/Mg++ in the buffer increased the half-life of tubulin colchicine binding activity from 11 hours to 90 hours (Weisenberg, et al., 1968), while the half life of tubulin decreased in the absence of Mg++-nucleotides, accompanied by changes in the tubulin circular dichroism pattern (Brown-Croom, et al., 1985). Taken together, these results demonstrated that Mg++-nucleotides stabilize the tubulin heterodimer.

*Localization of the Guanine Nucleotide Exchangeable Site*

A number of experimental approaches have been used to characterize the guanine nucleotide exchangeable binding site. The first attempts to map the precise site of interaction between tubulin and GTP used photolyzable analogs of GTP such as 8-N3-GTP. Tubulin heterodimer was incubated with 8-N3-GTP, the sample cross-linked, and the proteins separated by gel electrophoresis. In these experiments, 8-N3-GTP cross-linked to beta-tubulin (Geahlen and Haley, 1977). Since purified calf brain tubulin heterodimer polymerized with uncross-linked 8-N3-GTP, the analog was not inherently detrimental to normal heterodimer interactions. Experiments in a number of other systems demonstrated that unmodified GTP could be photo-crosslinked to GTP binding proteins by exposure to UV light in a process dependent on native protein (Maruta and Korn, 1981; Penefsky, 1977; Yue and Schimmel, 1977). Using this technique, the beta-tubulin subunit of the tubulin heterodimer was cross-linked to 32P-GTP, identifying this subunit as a critical component of the GTP exchangeable binding site on tubulin (Hesse, et al., 1985; Nath and Himes, 1986).
The GTP UV cross-linking technique was subsequently applied to precisely isolate the E-site on tubulin. Purified microtubules were cross-linked to $^{32}$P-α-GTP, the protein digested with trypsin, and the labeled fractions isolated and sequenced. The peptide associated with the most $^{32}$P-α-GTP label contained beta-tubulin amino-acids 155-174, although several additional peptides, including one containing residues 305 to 308 of alpha-tubulin, were also labeled. High molar ratios of polyclonal antisera generated against β-155-174 inhibited the incorporation of GTP into tubulin heterodimer and microtubule polymerization more efficiently than controls, pre-immune serum and antisera generated against the alpha-tubulin peptide containing residues 399-412. However, preimmune antisera and anti-α-tubulin antisera also affected GTP binding, albeit less well (Hesse, et al., 1987), raising concerns that the anti-beta-tubulin antibody inhibition was non-specific. One might expect absolute conservation of the GTP tubulin binding domain, since this interaction is likely critical for microtubule dynamics. Oddly, the peptide identified by these cross-linking experiments was not absolutely conserved. Other experiments using cross-linking technology have suggested the interaction between tubulin and GTP occurs near the carboxy-terminus of the protein, another poorly conserved region (Padilla, et al., 1993).

Cross-linking experiments have also been used to examine the interactions between microtubules and GTP. Cycled microtubules were polymerized in vitro and stabilized by the microtubule-stabilizing drug taxol. Fluorescent beads coated with GTP were incubated with the stabilized microtubules. Microscopic examination of the resultant microtubules revealed an interaction between a small number of GTP-coated beads an the microtubule plus ends. When the microtubules were cross-linked to radiolabeled GTP, the beta-tubulin polypeptide retained the label (Mitchison, 1993).

GTP cross-linking experiments have demonstrated that beta-tubulin is a necessary component of tubulin GTP binding. However, contrary to the interpretations of a number of investigators (Hesse, et al., 1985; Hesse, et al., 1987; Mitchison, 1993; Nath and Himes, 1986; Padilla, et al., 1993), these data do not address whether beta-tubulin is sufficient for GTP binding. In each experiment, GTP was cross-linked to tubulin heterodimer, not monomeric beta-tubulin. Alpha-tubulin may play a critical role in GTP binding.
The interactions between tubulin and guanine nucleotides at the E-site was also characterized using a second biochemical approach. Monomeric and dimeric beta-tubulin, produced from the in vitro translation of beta-tubulin genes in rabbit reticulocyte lysate preparations, was tested for GTP binding. The recovery of in vitro translated beta-tubulin in monomeric and dimeric forms from anion exchange columns was dependent on added nucleotides. The dimeric tubulin fraction had a four-fold higher capacity to bind GTP than monomeric beta-tubulin, in a Mg\(^{++}\) dependent manner. The measured GTP dimeric tubulin dimer dissociation constant was 10 nM for GTP and 40 nM for GDP, compared to 39 nM and 68 nM for beta-tubulin GTP and GDP, respectively. In the absence of Mg\(^{++}\), the dissociation constant for dimeric tubulin-GTP was 8100 nM and for beta-tubulin-GTP was 1600 nM (Farr, et al., 1990). These data suggested alpha-tubulin significantly contributed to tubulin GTP binding activity. However, only 20% to 35% of the monomeric beta-tubulin produced in vitro was assembly-competent (Yaffe, et al., 1988a) which could account for the binding differences between beta-tubulin and dimeric-tubulin.

Beta-tubulin does not have a standard GTP binding motif, but some similarities to other GTP binding proteins exist (Dever, et al., 1987; Sternlicht, et al., 1987). Using the in vitro translation GTP binding assay, the affects of mutations at sites in the purported GTP binding consensus sequence of chicken beta-tubulin were tested. Some mutations produced dramatic alterations in both beta-tubulin and dimer affinity for GTP (Farr and Sternlicht, 1992). Oddly, mutations at identical, conserved residues in the sole beta-tubulin of S. cerevisiae did not produce severe phenotypes (Reijo, et al., 1994). For example, the mutation D67N resulted in complete loss of in vitro GTP binding and the mutation E69Q modestly altered in vitro GTP binding. In S. cerevisiae, D67A combined with E69A was wildtype by a number of growth assays. No mutation with in vitro GTP binding alterations was lethal in vivo (Reijo, et al., 1994). Although the mutations were not identical, the results in S. cerevisiae urge caution in interpreting the results of the in vitro GTP binding assays.

GTP binding and hydrolysis have been recently characterized in S. cerevisiae (Davis, et al., 1993). Using a modification of a yeast tubulin purification method (Barnes, et al., 1992), yeast tubulin GTP binding affinity was calculated as \(K_a = 1.7 \times 10^7\) M\(^{-1}\) with a stoichiometry of ~1 mol GTP:1 mol dimer.
(Davis, et al., 1993). Unfortunately, the tubulin was not completely pure. Since tubulin protein levels were measured spectrophotometrically, and $^3$H-GTP binding was measured using scintillation counters, contaminants that bind GTP or affect tubulin-GTP binding cannot be excluded. Davis and colleagues determined the purported GTP hydrolysis site in beta-tubulin as $^{103}$Lys-Gly-His-Tyr-Thr-Glu-Gly$^{109}$, determined from members of the GTPase superfamily as Gly-X-X-X-X-Gly-Lys. Mutations at the Tyr residue were homozygous lethal. In heterozygous strains, these mutations adversely affected GTP hydrolysis, and higher concentrations of tubulin were required to assemble microtubules. Mutations at the conserved $^{103}$Lys were not lethal and did not alter rates of GTP hydrolysis (Davis, et al., 1994). These results suggest that the phenotypic mutations may have distal consequences on protein structure, rather than direct effects on tubulin GTP hydrolysis.

Experiments in *S. cerevisiae* have failed to resolve whether beta-tubulin is necessary and sufficient for tubulin GTP binding. To date, no mutations in alpha-tubulin with altered GTP binding phenotypes have been identified. In chapter three of this dissertation, I will describe experiments examining the GTP binding properties of relatively pure protein preparations enriched for monomeric alpha- or beta-tubulin. The results of these experiments, which rely on UV cross-linking techniques, demonstrate that both alpha- and beta-tubulin subunits contribute to GTP binding activity.

**Post-Translational Modification of Tubulin**

Alpha- and beta-tubulin molecules undergo several types of post-translational modifications *in vivo*. Briefly, alpha-tubulin is tyrosinated in the heterodimer and detyrosinated in the polymer. The implications of tyrosination and detyrosination on microtubule function are unclear. Detyrosinated tubulin is preferentially localized to stable microtubules, but evidence suggests they do not contribute to microtubule stability (MacRae and Langdon, 1989). Alpha-tubulin is also acetylated, at lysine 40, and glutamylated near the carboxy-terminus (Edde, et al., 1990; MacRae and Langdon, 1989). The physiological relevance of these modifications has yet to be established. The introduction of a non-acetylatable alpha-tubulin in *Chlamydomonas*, which had no phenotypic
consequences (Kozminski, et al., 1993), shows that at least some of the post-translation modifications of tubulin are not essential for microtubule function.

**Microtubule Structure**

**Formation of Microtubules**

Microtubules are complex polymeric structures with alpha- and beta-tubulin heterodimers the basic subunit of the protofilament. Microtubule are composed of 8 to 19 tubulin protofilaments, with 12 to 15 typical, that interact together to form a sheet (Wade and Chretien, 1993). The sheet of protofilaments closes into a tube producing the basic structure that comprises a number of cytoplasmic and mitotic microtubule polymers (Simon and Salmon, 1990). More complex microtubules, such as those found in flagella, form a “figure eight”, composed of one complete tubule containing 13 protofilaments, named the A-tubule, and one incomplete tubule containing 10 or 11 protofilaments, named the B-tubule (Amos and Klug, 1974).

The microtubule protofilament is polar, exhibiting characteristic polarity within a given microtubule structure. "Minus' ends of microtubules assemble more slowly, interact preferentially with nucleators, and are usually located proximal to the cell body. The "plus" ends assemble and disassemble quickly, and are typically distal to the cell body (Bergen and Borisy, 1980; Borisy, 1978; Euteneur and McIntosh, 1981; Heidemann and McIntosh, 1980; Kirschner, 1980). All the microtubules of flagella, for example, have their plus ends at the cell periphery (Bergen and Borisy, 1980).

Purified alpha- and beta-tubulin heterodimer subunits, in the presence of Mg++/GTP, glycerol, and calcium chelators, polymerize to form normal microtubules (Lee and Timasheff, 1975). These data show that all the structural components required to form microtubules are contained within the tubulin heterodimer. Alpha- and beta-tubulin polypeptides must be capable of interacting with one another to form protofilaments independent of interacting proteins.

A number of structural studies have attempted to map the precise interactions between tubulin heterodimers that form microtubules. These include X-ray diffraction analysis and electron microscopic analysis (Amos and Klug, 1974; Beese, et al., 1987; Crepeau, et al., 1978; Downing and Jontes,
From the data obtained, several models have been proposed. The two models most commonly encountered in textbooks are the A-lattice model and the B-lattice model, which were thought to correspond to the A- and B-tubules of the axoneme, respectively (Amos and Klug, 1974).

The A-lattice model predicts that microtubule protofilaments interact with one another to form a smooth helical structure with no seams. In the B-lattice model, the interactions of alpha- and beta-tubulin between protofilaments in the microtubule sheet are smooth and precise, but the model predicts a “seam” where alpha- and beta-tubulin encounter one another as the microtubule sheet closes into a tube (Amos and Klug, 1974; Beese, et al., 1987; Crepeau, et al., 1978; Downing and Jontes, 1992; Simic-Krstic, et al., 1989; Song and Mandelkow, 1993; Wade and Chretien, 1993).

It has been extremely difficult to test which of these models occurs in vivo, in the axoneme, in cytoplasmic, and in mitotic microtubules. Alpha- and beta-tubulin are so similar to one another that they are indistinguishable, given the resolution of currently available structural techniques. As a consequence, it has been impossible to map the precise interactions between the heterodimer subunits in the microtubule lattice predicted by each model.

Recently, one to one molar binding between the tubulin heterodimer and a truncated form of the microtubule-associated protein kinesin was discovered (Harrison, et al., 1993). Kinesin was cross-linked to beta-tubulin polypeptides in the protofilament and soluble pool (Song and Mandelkow, 1993). Since the kinesin molecule produced a characteristic change in the diffraction pattern for microtubules (Harrison, et al., 1993), it allowed researchers to resolve alpha- and beta-tubulin for the first time.

Microtubules grown from the ends of axonemes open into a flattened sheet. These microtubules were labeled with kinesin and the diffraction pattern characterized. Microtubules grown from both the axonemal A- and B-tubules exhibited the same diffraction pattern, and it was consistent with the B-lattice model. The diffraction studies also enabled researchers to determine that beta-tubulin was at the minus end and alpha-tubulin was at the plus end of the microtubule polymer (Song and Mandelkow, 1995).
Introduction

One interesting prediction of the B-lattice model is that axonemal microtubules with defective interactions at the seam should produce S-shaped (not ω-shaped) microtubules in cross-section. This is precisely the phenotype observed \textit{in vivo} in \textit{D. melanogaster} strains carrying the B2t\textsuperscript{8} mutation, a glutamic acid to lysine mutation at highly conserved position 288 of the testes-specific beta-tubulin of \textit{D. melanogaster} (Fuller, \textit{et al.}, 1987). Microtubules nicked with proteases also produced a similar phenotype (Mandelkow, \textit{et al.}, 1985). These data lend credence to the diffraction pattern studies that predict alpha- and beta-tubulin interact according to the B-lattice model.

\textbf{Polymerization and Depolymerization}

The microtubule polymer is a dynamic structure, rapidly responding to cellular cues. The transition from interphase into mitosis, for example, requires a dramatic reorganization of microtubules to form the mitotic spindle. The major tenet of the Dynamic Instability Hypothesis (Kirschner and Mitchison, 1986; Mitchison and Kirschner, 1984) is that within a population of microtubules at steady state, some microtubules are growing while others are undergoing an abrupt catastrophic collapse.

Tubulin heterodimers are added to the ends of the microtubule polymer in a concentration dependent manner. Theoretically, this can occur at each end of the microtubule polymer, since both the plus and minus ends of the polymer can acquire new subunits. However, one prediction of the dynamic instability hypothesis is that microtubules produced from nucleators would be favored over free microtubules, since one end of the dynamic microtubules is stabilized. This result was confirmed by examining individual microtubules in the presence of nucleators in vitro. The majority of microtubules originated from nucleating sites (Mitchison and Kirschner, 1984).

Microtubules exhibited the properties of dynamic equilibrium \textit{in vivo}. Early research using light microscopy identified dynamic microtubules \textit{in vivo}, rapidly growing and collapsing (Inoue and Sato, 1967). More recent experiments, using labeled microtubules or computer enhanced microscopy, have identified and characterized the rates of this phenomena \textit{in vivo} (Cassimeris, \textit{et al.}, 1988).
A second prediction of the Dynamic Instability model is that growing microtubules contain a "cap" of GTP that protects the microtubule from disassembly. As polymerization occurs, GTP within the polymer is converted to GDP. However, GTP is maintained at the ends of the microtubule, possible due to higher rates of subunit addition than GTP hydrolysis. These microtubules are stable, as long as the GTP-tubulin at the end of the polymer is present. Loss of the protective GTP cap results in catastrophe. Models predicting the precise location and composition of the GTP cap have been advanced (for example: (Bayley, et al., 1990)), and experiments predict the size of the GTP cap must be exceedingly small, since it has defied detection from a number of experimental approaches (Stewart, et al., 1990; Walker, et al., 1991). However, the presence of a tubulin GTP cap has yet to be demonstrated.

Role of GTP Hydrolysis

The Dynamic Instability Model predicts that microtubules are capable of rapid growth and rapid collapse. Since the process is not at equilibrium, it must require a source of energy. Mitchison and Kirschner predicted that the source of this energy would be tubulin GTP hydrolysis.


These data suggest GTP hydrolysis does not provide the energy for tubulin polymerization. However, growing evidence from experiments with GTP analogs suggest GTP hydrolysis provides energy required for tubulin
Introduction

Microtubules produced in the presence of slowly hydrolyzable and non-hydrolyzable analogs of GTP are more stable. Treatments with Ca++, the cold, or depolymerizing drugs less effectively depolymerized these microtubules (Arai and Kaziro, 1977; Hyman, et al., 1992; Mejillano and Himes, 1991; O'Brien and Erikson, 1989; Sandoval, et al., 1977; Wehland and Sandoval, 1983; Weisenberg and Deery, 1976).

Examination of microtubules polymerized with GTP by electron microscopy showed moiré and stripe patterns characteristic of 13, 14, or 15 protofilaments. However, the GTP analog GMPCPP was almost entirely 14 protofilaments. The characteristic spacing pattern for heterodimer subunits also increased in size with the GTP analog. These data suggested that GTP hydrolysis is accompanied by a corresponding tightening of the microtubule pattern, possibly contributing to depolymerization (Hyman, et al., 1995).
Conclusions

The microtubule lattice is an intricate, delicate, and complex structure, critically important for a number of cellular functions. Years of research, with a broad array of approaches, has tremendously advanced our understanding of tubulin biochemistry and structure. Never-the-less, a number of major mysteries remain.

First, the microtubule heterodimer and the individual alpha- and beta-tubulin subunits have not been crystallized. As a consequence, precise understanding of microtubule structure function is not yet available. Second, the precise role of GTP hydrolysis in microtubule polymerization, and the location of the "GTP cap" or a functional alternative require additional experimentation. Third, the basic functional differences, if any, between the alpha- and beta-tubulin polypeptides are not known because it has not been possible to produce these polypeptides in sufficient quantities for biochemical analysis.

This dissertation describes two basic experimental approaches, one genetic and one biochemical, that address questions about the structural and biochemical roles of the individual alpha- and beta-tubulin subunits. Chapter Two describes experiments characterizing a mutation in S. Cerevisiae beta-tubulin. An identical change in the testes-specific beta-tubulin of D. melanogaster produced dramatic phenotypic consequences. The rationale for this approach was to identify critical residues within the beta-tubulin primary sequence, and, using genetic suppresser analysis, critical residues within alpha-tubulin, required for tubulin heterodimer formation or microtubule polymerization.

Chapters Three and Four describe a procedure to produce protein preparations highly enriched for alpha- and beta-tubulin monomer in vitro. Tubulin produced using this procedure was of sufficient concentration and purity to perform biochemical experiments allowing the characterization of the individual tubulin chains, and the contribution of each to tubulin GTP. The results of this analysis have important repercussions for models of tubulin GTP binding and structure.
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Chapter Two:

A codon change in β-tubulin which drastically affects microtubule structure in Drosophila fails to produce a significant phenotype in yeast

Abstract

The relative uniformity of microtubule ultrastructure in almost all eukaryotic cells is thought to be a consequence of the conserved elements of tubulin sequence. In support of this idea, a mutation in a β-tubulin gene of D. melanogaster, occurring at a highly conserved position, produces U-shaped microtubules, suggesting a defect in either nucleation or packing during assembly (Fuller, et al., 1987; Rudolph, et al., 1987). Surprisingly, we find that introducing the same mutation into the sole β-tubulin gene of S. cerevisiae has virtually no consequences for microtubule structure or function in that organism.
Chapter Two

Introduction

In almost all eukaryotic cells, and in both nuclear and cytoplasmic cytoskeletal organelles, the ultrastructure of microtubules is highly conserved. Almost certainly, the constant elements of microtubule structure are specified by the regions of highly conserved sequences found in the two major protein components of microtubules, the α- and β-tubulins (Cleveland and Sullivan, 1985).

One approach to analyzing the functional role of tubulin sequence is to examine the consequence of mutations. A large number of mutations in both α- and β-tubulin have been identified which affect microtubule assembly. A particularly intriguing example of such a mutation is found in the testes-specific β-tubulin gene of *Drosophila melanogaster*. The B2tδ mutation encodes a lysine instead of a glutamic acid at position 288 (Rudolph, *et al.*, 1987). A search of GenBank identifies 46 β-tubulin sequences which have glutamic acid at this position. Three exceptions - *Gallus gallus* B3 (Sullivan, *et al.*, 1986), *Leishmania mexicana* (Fong and Lee, 1988), and *Homo sapiens* M40 (Hall, *et al.*, 1983) - have aspartic acid, a highly conserved substitution. In *D. melanogaster*, the B2tδ mutation dramatically disrupts the most conserved of all microtubule properties, assembly into polymers with a circular cross-section. Instead, the homozygous mutant displays polymers which have failed to close. The cross-sections are U-shaped or even S-shaped, suggesting a packing or nucleation defect (Fuller, *et al.*, 1987; Rudolph, *et al.*, 1987). Homozygous individuals are sterile, and all the microtubule organelles in the testes are abnormal (Fuller, *et al.*, 1987).

We describe here the introduction of this glutamic acid to lysine mutation at position 288 of *TUB2*, the single *S. cerevisiae* β-tubulin gene. We were motivated to make this mutant in order to use yeast molecular genetics to understand in detail the interactions which make this residue so functionally critical. We report here that this mutation fails to disrupt microtubule function in yeast in any significant way.
Materials and Methods

Plasmid constructions

pRB429, a yeast vector containing codons 5 to 457 of TUB2, (Huffaker, et al., 1988) was digested with EcoRI and Hind3 to produce a 1.1kb fragment containing codons 5 to 392 of the TUB2 gene. Using oligonucleotide directed mutagenesis (Muta-gene from BioRad), the mutation was introduced into this subcloned fragment with a 21-mer oligonucleotide (ACT GTC CCT AAG TTA ACA CAG) encoding a lysine at amino-acid position 288 (underlined; the wild type sequence is GAA). The presence of the mutation in the EcoRI/Hind3 fragment was confirmed by sequencing. The mutant β-tubulin fragment was then reintroduced into pRB429 to produce pWK60. pWK60 is identical to pRB429 except for the double point mutation at codon 288; both contain a URA3 selectable marker, and lack a yeast origin of replication as well as the sequence upstream of codon 5 of TUB2. This mutant allele of TUB2 is called tub2-592. Maintenance of the selectable marker requires that the plasmid be integrated into the yeast genome so that only one β-tubulin will be expressed at the TUB2 locus (Figure 2.1A).

Strains and media

Genotypes of strains used are listed in Table 1. To generate diploid strains FSY301 through FSY306, FSY129 was transformed with pWK60. The transformation efficiencies were similar to those seen in parallel transformations with pRB429. URA+ transformants containing and expressing the plasmid-borne allele, as assayed by Southern blotting and immunofluorescence, were grown on medium containing 5-fluoro-orotic acid (5-FOA) to select for strains which had undergone plasmid excision Figure 2.1B; (Boeke, et al., 1984) These strains were assayed for the presence of the mutation, again by both immunofluorescence and Southern blot.

To generate haploid strains FSY321 and FSY322, FSY127 was transformed with pRB429 and pWK60, respectively. The URA+ transformants were tested by immunofluorescence and Southern blot.
Chapter Two

FSY307 through FSY310 were haploid progeny from a single tetrad produced by sporulating FSY300. FSY311 through FSY314 were progeny from a single tetrad produced by sporulating FSY303. FSY315, FSY316, and FSY320 are haploid segregants produced from sporulating FSY304, FSY306, and FSY120, respectively. FSY317 through FSY319 were produced by crossing the haploid strains previously described. All of these strains were characterized by both by immunofluorescence and Southern blot, as above.

SCD media were synthesized as described previously (Katz, et al., 1990). Benomyl was made in a 10mg/ml stock in DMSO and added to SCD media (Katz and Solomon, 1988). Other media were as described in (Sherman, et al., 1986).

**Genetic Techniques and Transformation**

Yeast genetic methods were as previously described in (Sherman, et al., 1986). Transformations were done by the lithium acetate method and sporulations were carried out in 1% potassium acetate solution.

**Immunofluorescence**

Cells were fixed, permeabilized, and stained with anti-sera specific for yeast β-tubulins as described previously (Katz and Solomon, 1988). The original transformations with pWK60 were performed on FSY129 (Katz and Solomon, 1988), a diploid strain homozygous for the "tailless" allele of TUB2, tub2-590, lacking the C-terminal 12 amino acids (Katz and Solomon, 1988). This gene product is not recognized by antiserum 206, which was raised against a peptide containing those amino acids, but is recognized by antiserum 339, which was raised against the penultimate 12 amino acids (Katz and Solomon, 1988). Since the mutation at codon 288 was closely associated with the normal C-terminus, transformants were screened first with antiserum 206 to identify candidates, which were then analyzed further by Southern blotting and direct protein analysis (see below).
Protein Blotting

Protein from the haploid strains FSY321(TUB2) and FSY322(tub2-592) was harvested as described (Katz and Solomon, 1988), and analyzed by two-dimensional electrophoresis (O’Farrell, 1975; Pallas and Solomon, 1982). Samples were transferred to nitrocellulose electrophoretically (Dinsmore and Sloboda, 1989; Tobin, et al., 1979). In order to determine the position of $\beta$-tubulin, blots were first probed with anti-$\beta$-tubulin antiserum, 206, and $^{125}$I-labeled Protein A (New England Nuclear). After exposure, blots were reprobed with anti-$\alpha$-tubulin antiserum, 345, (Schatz, et al., 1988), and alkaline phosphatase-conjugated goat anti-rabbit IgG, which recognizes both 206 and 345, and visualized in a color reaction (BioRad).

Growth Rate Analysis

Duplicate aliquots from cultures in logarithmic growth were quantitated in a hemacytometer. At the end of each growth assay, cells from the cultures were examined by immunofluorescence staining and Southern blot to confirm their $\beta$-tubulin genotype.

Southern Blots

Total cellular DNA was prepared (Holm, et al., 1986) and digested with EcoRI (New England Biolabs), run on 0.7% agarose TBE gels, and blotted onto nylon (BioTrace, Gelman Sciences) according to standard techniques (Maniatis, et al., 1989) or with 0.4M NaOH (Reed and Mann, 1985). Blots were UV crosslinked (0.12 joules; Stratalinker UV Crosslinker, Stratagene) then pre-hybridized twenty minutes to one hour in 5X SSC, 0.7% SDS, 0.05 M NaPhosphate buffer, and 10X Denhardtts, and hybridized in the same solution, containing the probes, overnight at 37°C. The blots were washed three times at 37°C, in 5X SSC/1% SDS with a final wash at 42°C in 1X SSC, (Church and Gilbert, 1984; Devlin, et al., 1988). The probes, 21-mer oligonucleotides containing wildtype and mutant sequences, were synthesized in the Biopolymers Laboratory, Center for Cancer Research, MIT, and labeled using [g-32P]ATP (ICN) and poly-nucleotide kinase (Maniatis, et al., 1989).
Table 1. Yeast strains and Plasmids used

<table>
<thead>
<tr>
<th>strain or plasmid</th>
<th>Relevant genotype or genes</th>
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<tbody>
<tr>
<td>FSY120</td>
<td>MATα, his4-619/+, leu2-3,112/leu2-3,112, lys2-801/+, ura3-52/ura3-52, TUB2/TUB2</td>
</tr>
<tr>
<td>FSY129</td>
<td>MATα, his4-619/+, leu2-3,112/leu2-3,112, lys2-801/+, ura3-52/ura3-52, tub2-590/tub2-590</td>
</tr>
<tr>
<td>FSY300-FSY306</td>
<td>FSY129 transformed with pWK60 and then grown on 5-FOA.</td>
</tr>
<tr>
<td>FSY307-FSY310</td>
<td>Haploid segregants from one tetrad of FSY300</td>
</tr>
<tr>
<td>FSY311-FSY314</td>
<td>Haploid segregants from one tetrad of FSY303</td>
</tr>
<tr>
<td>FSY315</td>
<td>Haploid segregant from FSY304, tub2-592</td>
</tr>
<tr>
<td>FSY316</td>
<td>Haploid segregant from FSY306, tub2-592</td>
</tr>
<tr>
<td>FSY317</td>
<td>Haploid segregant from FSY120, TUB2</td>
</tr>
<tr>
<td>FSY127</td>
<td>MATα, leu2-3,112, lys2-801, ura3-52, tub2-590</td>
</tr>
<tr>
<td>FSY321</td>
<td>FSY127 transformed with pRB429, TUB2</td>
</tr>
<tr>
<td>FSY322</td>
<td>FSY127 transformed with pWK60, tub2-592</td>
</tr>
<tr>
<td>FSY317</td>
<td>FSY314 x FSY315, tub2-592/tub2-592</td>
</tr>
<tr>
<td>FSY318</td>
<td>FSY314 x FSY316, tub2-592/tub2-592</td>
</tr>
<tr>
<td>FSY319</td>
<td>FSY314 x FSY308, tub2-592/tub2-592</td>
</tr>
</tbody>
</table>

Plasmids

<table>
<thead>
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<th>plasmid</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>pRB429b</td>
<td>Codons 5-457 of TUB2, URA3</td>
</tr>
<tr>
<td>pWK60</td>
<td>Codons 5-457 of tub2-592, URA3</td>
</tr>
</tbody>
</table>

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a All strains are derived from S288C strains provided by D. Botstein and G. Fink (Massachusetts Institute of Technology).
b Source: D. Botstein.
Results

Construction of a mutant TUB2 gene

The two base changes used to alter codon 288 of TUB2 from glutamic acid to lysine were generated in the 1.1kb EcoRI-HindIII fragment of TUB2 by oligonucleotide generated mutagenesis and confirmed by sequencing (see Materials and Methods). We subcloned the mutant fragment into pRB429, a yeast vector containing codons 5 to 457 of TUB2 and a URA3 selectable marker but lacking a replication origin. The plasmid bearing the mutation was named pWK60 and the mutant β-tubulin allele was designated tub2-592.

_tub2-592_ transformants contain the glu to lys change at position 288

We transformed the diploid yeast strain FSY129 with pWK60 and with pRB429, the wildtype control, directing integration to the TUB2 locus by linearizing the plasmid at a KpnI site in the amino-terminal portion of β-tubulin upstream of the tub2-592 mutation (Figure 2.1A). The transformants were selected by their ability to grow on media lacking uracil. Similar transformation frequencies were obtained with both wildtype and mutant plasmids.

FSY129 is homozygous for tub2-590, which encodes an allele of β-tubulin lacking the C-terminal 12 amino acids. That protein is not recognized by antiserum (206) specific for those amino acids (Katz and Solomon, 1988). By screening URA+ transformants using immunofluorescence staining with 206 antisera, we could detect the full-length β-tubulin carboxy-terminus, which is closely linked to the tub2-592 mutation, in strains expressing the transformed copy of β-tubulin. Four out of ten URA+ transformants tested were 206 positive.

Since these strains contained a partial copy of tub2-590 (Figure 2.1A), the possibility of gene conversion to correct the mutation, even in haploid progeny, was a concern. Therefore, three 206 positive transformants were grown on 5-FOA, a drug which kills cells expressing the URA3 gene. Elimination of the sequence introduced on the plasmid by homologous recombination can occur upstream of the tub2-592 mutation, producing the _truncated_ tub2-590 allele with
Figure 2.1. (A) Chromosomal integration of tub2-592, introduced into tub2-590/tub2-590 strains by the yeast transforming vector pWK60. (B) Excision of the vector DNA by growth on 5-FOA. Symbols: tub2-592 DNA, including 3' non-coding sequence; the glutamic acid to lysine substitution at position 288 of β-tubulin is represented by *; the jagged line indicates the 5' deletion of the tub2 insert on the plasmid; tub2-590 chromosomal DNA, including 3' non-coding sequence; plasmid-derived URA3 sequence; chromosomal DNA flanking the tub2 region and sequence derived from the plasmid backbone.
the wildtype codon at 288, or downstream of the mutation, yielding a diploid strain which stably maintains the *tub2-592* mutation and the wildtype carboxy terminus in one copy of β-tubulin (Figure 2.1B). Twelve out of twenty strains obtained by this selection were positive for the wildtype carboxy-terminus in an immunofluorescence screen and therefore were likely to contain the closely-linked mutation at codon 288.

To confirm the presence of the mutation in these heterozygous strains, we tested them by probing genomic Southern blots with oligonucleotides containing either wildtype or mutant sequence, as described in Materials and Methods. DNA from ten strains, seven originally transformed with pWK60 and three with pRB429, was digested with the restriction enzyme *EcoRI*, which yields a 1.6kb fragment in the *TUB2* coding region, and probed with mutant and wildtype oligonucleotides in parallel blots. As expected, pWK60 transformants showed hybridization with both probes, indicating one copy each of *tub2-590* and *tub2-592*, while pRB429 transformants reacted only with wildtype probe (data not shown).

We used two-dimensional gel electrophoresis to analyze β-tubulin proteins of the haploid strains FSY321 and FSY322, which by immunofluorescence and Southern blot criteria were shown to contain *TUB2* and *tub2-592* genes, respectively. Protein blots were probed with 206 antiserum to identify 3-tubulin and with 345 antiserum which recognizes both α-tubulin proteins (Figure 2.2). The β-tubulin from the mutant strain, FSY322, focused to a more basic position than the β-tubulin protein from the wildtype strain, as predicted for the Tub2-592 gene product.

**Phenotypic Analysis of *tub2-592***

We tested several strains bearing *tub2-592* to determine the consequences of this mutation for microtubule-dependent functions.

**Growth, sporulation, germination, and mating.**

Heterozygous *tub2-592* strains grew at rates indistinguishable from wildtype strains at 30°C. They sporulated at frequencies similar to wildtype strains (averaging 20-30%) and the haploid progeny from 62 tetrads segregated 4:0 for viability and 2:2 for *HIS* and *LYS* auxotrophic markers.
Figure 2.2. Western blots of two-dimensional gels from whole-cell extracts of haploid strains bearing the TUB2 allele (FSY321) or the tub2-592 allele (FSY322). An arrow indicates the spot recognized by anti-β-tubulin antiserum, 206. The two more basic spots recognized by anti-α-tubulin antiserum, 345, represent TUB1 and TUB3.
Ten tetrads were assayed for segregation of the wild type carboxy terminus of TUB2 by immunofluorescence with antiserum 206; all showed 2:2 segregation of this marker. Two tetrads were selected for analysis of segregation of the mutant sequence. Both showed 2:2 segregation as assayed by Southern blot and by staining with antibody 206 for the carboxy terminus associated with the tub2-592 gene (Figure 2.3A). These results indicate that haploid strains expressing only the tub2-592 allele of β-tubulin are viable and require no unlinked suppressors to survive.

Diploids homozygous for tub2-592 (FSY317, 318 and 319; see Table 1) were characterized by Southern blots to confirm the presence of the tub2-592 mutation and the absence of the wild-type TUB2 allele (Figure 2.3B). The tub2-592 diploids grew at wildtype rates at 30°C. They sporulated at frequencies similar to wild-type strains, and the haploid progeny segregated 4:0 for viability.

Temperature and cold sensitivity.

We grew haploid progeny from heterozygous tub2-592 strains on complete media at 11°C, 15°C, and 37°C to determine if they were cold or temperature sensitive (Huffaker, et al., 1988; Schatz, et al., 1988). tub2-592 haploids grew at the same rate as their tub2-590 sisters, and at the same rate as TUB2 haploid strains, at all of these temperatures (data not shown).

Benomyl sensitivity.

We could detect only one phenotypic consequence of the mutation, modest supersensitivity to benomyl, a microtubule depolymerizing drug (Kilmartin, 1981; Neff, et al., 1983; Thomas, et al., 1985). tub2-592 strains grew more slowly on media containing benomyl than TUB2 strains but grew faster than tub2-590 strains, which carry a truncation in the carboxy terminus of β-
tubulin and are benomyl super-sensitive (Katz and Solomon, 1988). The growth rates for strains bearing \textit{TUB2}, \textit{tub2-590}, and \textit{tub2-592} at one concentration of benomyl (40\textmu g/ml) in liquid medium are shown in Table 2.

**Table 2.** Growth rates of wildtype and mutant strains in 40\textmu g/ml benomyl.

<table>
<thead>
<tr>
<th>Strain*</th>
<th>Genotype</th>
<th>Doubling Time**</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSY311</td>
<td>tub2-592</td>
<td>3.6 hours</td>
</tr>
<tr>
<td>FSY312</td>
<td>tub2-590</td>
<td>7.1 hours</td>
</tr>
<tr>
<td>FSY313</td>
<td>tub2-590</td>
<td>5.7 hours</td>
</tr>
<tr>
<td>FSY314</td>
<td>tub2-592</td>
<td>4.3 hours</td>
</tr>
<tr>
<td>FSY320</td>
<td>TUB2</td>
<td>2.0 hours</td>
</tr>
</tbody>
</table>

*FSY311, FSY312, FSY313, and FSY314 were haploid segregants from one tetrad.

** Doubling times were derived from plots of increase in cell number with respect to time.
Figure 2.3. Southern blots probed with 21-mer oligonucleotides bearing the glutamic acid to lysine mutation or the wildtype sequence. (A) Lanes 1-4 represent DNA derived from haploid strains FSY307-FSY310, respectively, probed with mutant oligomers. Lanes 5-8, run on parallel blots, represent DNA from haploid strains FSY307-FYS310, respectively, and were probed with wildtype oligomers. The presence of sequences hybridizing to the mutant oligonucleotide probe corresponds to the presence of the full-length C-terminus of the b-tubulin protein ("206 staining"). (B) DNA from diploid strains FSY317, FSY318, and FSY319, generated by crossing tub2-592 haploid strains, and FSY120 a TUB2/TUB2 strain. Lanes 1-4 were probed with mutant oligomers and lanes 5-8 were probed with wildtype oligomers.
Discussion

Analysis of $\alpha$- and $\beta$-tubulins genes from protists, fungi, plants, and animals demonstrate a strikingly high degree of sequence conservation. The pressure to conserve the identity of specific residues is most simply explained if they contribute to conserved aspects of quaternary structure, such as the formation of tubulin dimers, the details of microtubule ultrastructure, and interactions with microtubule binding proteins. Crystal structures, which would permit analyses of the role of primary sequences in intermolecular interactions, are not yet available for the tubulins. An alternative approach would exploit tubulin genes in accessible genetic organisms, and the well-characterized and readily assayable functions of microtubules, to produce a structure-function analysis.

It was for the latter purpose that we chose to construct an allele of the \textit{S. cerevisiae} $\beta$-tubulin gene, \textit{TUB2}, bearing a substitution of lysine for glutamic acid at position 288. A survey of cloned $\beta$-tubulin genes revealed that 46 of them have glutamic acid at this position. The three exceptions have aspartatic acid. This record suggests a functional role for a negative charge at this position in the protein. However, evolutionary conservation is not itself compelling evidence for a functional role. That point has been made clearly by the introduction of amino acid substitutions into cytochrome c at similarly conserved positions, substitutions which caused little or no perturbation in protein function (Das, \textit{et al.}, 1988; Holzschu, \textit{et al.}, 1987). A more direct and independent test of function for Glu-288 is provided by analysis of tubulin mutants in \textit{D. melanogaster}. The $B2t^8$ allele of the testes-specific $\beta$-tubulin gene produces tubulin polymers which do not provide wild type function, and which are morphologically abnormal - U-shaped cross-sections, or S-shaped cross-sections containing about double the normal number of protofilaments. The glutamic acid at position 288 of $B2t^8$ is replaced by a lysine (Fuller, \textit{et al.}, 1987; Rudolph, \textit{et al.}, 1987). The results suggest that this conserved anionic residue is important for packing or nucleation.

Here we show that \textit{tub2-592}, the mutant allele of \textit{TUB2} containing this same substitution of a cationic residue for an anionic one, is sufficient to support
yeast microtubule functions in a manner essentially indistinguishable from the wild type allele. It is possible that the disparity between the results in yeast and flies is due to special properties of either organism - for example, that yeast is not a metazoan or that microtubule function in testes requires organization of highly specialized organelles. But this explanation is made less likely by several considerations. First, the ultrastructural defect conferred by the $B2t^8$ allele is not confined to those microtubule organelles unique to testes. Instead, all of the microtubule organelles in testes - including cytoplasmic arrays and spindles - are affected. Indeed, tubulin isolated from homozygous $B2t^8$ mutants and allowed to polymerize in vitro produces abnormal sheets similar to the in vivo structures (Fuller, et al., 1987; Rudolph, et al., 1987). Finally, the conspicuous defect in microtubule assembly displayed by this mutant protein affects a conserved structural property of all microtubules - polymerization to form the microtubule itself - one which should come early in any morphogenetic pathway of microtubule organization into specialized organelles. What remains is that conserved microtubule functions represented by proper assembly are specified sufficiently by other residues in either the yeast $\beta$-tubulin itself, or in the $\alpha$-tubulins with which it interacts. Sorting out which residues are involved will require the sort of structural information which this experiment was initially designed to generate.

This essentially negative result underscores again the influence of context on the function of protein sequences. It also illustrates the need for caution in extrapolating function from sequence, and in interpreting either mutational or chimeric analyses which produce loss of function.
Acknowledgments

We thank Minx Fuller (Stanford) for suggesting this experiment; Bob Sauer and David Litwack for comments on the manuscript; Mary O'Connell for assistance in labeling oligonucleotides; and the members of our laboratory for valuable assistance throughout the development of this work. Oligonucleotide synthesis was performed at the Biopolymers Laboratory, Howard Hughes Medical Institute, Massachusetts Institute of Technology.

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Chapter Three

GTP Binding to Tubulin

Involves both $\alpha$– and $\beta$–Tubulin

Abstract:
The precise contribution of alpha- and beta-tubulin to tubulin GTP binding is poorly understood. We developed a procedure to separate native alpha- and beta-tubulin from heterodimeric complexes using non-ionic detergents. The resultant alpha- or beta-tubulin was tested for its ability to bind GTP using a photo-cross-linking assay. Native levels of GTP binding were restored when equimolar amounts of each subunit were present, demonstrating both chains contribute to tubulin GTP binding.
Chapter Three

Introduction

Microtubules are dynamic structures, rapidly adding or losing tubulin heterodimers from the end of the polymer, a phenomenon known as dynamic instability (Kirschner and Mitchison, 1986; Mitchison and Kirschner, 1984). Previous studies have identified GTP as a critical component of microtubule dynamic instability. Tubulin containing GTP-Mg\(^{++}\) promotes the assembly of tubulin heterodimers into polymers while tubulin GDP-Mg\(^{++}\) is unstable in the tubulin polymer (Weisenberg and Deery, 1976). Hydrolysis of GTP to GDP in tubulin heterodimers appears to be important for the depolymerization of microtubules since tubulin heterodimers containing poorly hydrolyzable GTP analogs do polymerize, but depolymerize slowly (Hyman, et al., 1992).

The interaction between the tubulin heterodimer and GTP has been characterized in vitro. There are two tubulin GTP binding sites in the tubulin heterodimer, one exchangeable and one non-exchangeable (Weisenberg, et al., 1968). GTP can be covalently cross-linked to the tubulin heterodimer by exposure to UV light. In these experiments, GTP is associated with the \(\beta\)-tubulin subunit, as assayed by migration during acrylamide gel electrophoresis (Geahlen and Haley, 1977; Hesse, et al., 1987; Nath and Himes, 1986). The \(\beta\)-tubulin subunit does not contain a typical GTP binding consensus site (Dever, et al., 1987; Sternlicht, et al., 1987). Mutations in the purported GTP binding site of the sole \(\beta\)-tubulin of S. cerevisiae adversely affect GTP binding and hydrolysis (Davis, et al., 1994). While these data suggest \(\beta\)-tubulin plays a critical role in tubulin GTP binding, they do not address or preclude the contributions of \(\alpha\)-tubulin. We assayed GTP binding in vitro in protein preparations enriched for S. cerevisiae monomeric \(\alpha\)- or \(\beta\)-tubulin to determine the contribution to GTP binding of each of the tubulin primary subunits.
Materials and Methods

Monoclonal Antibody Generation

The two peptides used to generate monoclonal antibodies were NH2-CADSYAEEE-COOH (TUB1C peptide; Biopolymers Laboratory, Center for Cancer Research, M. I. T.) and NH2-CQNQDEPITENFE-COOH (TUB2C peptide; Clontech, Palo Alto, California). The peptides were coupled to Keyhole Limpet Hemocyanin (KLH, Sigma Chemical) using standard methods (Harlow and Lane, 1988). Hybridomas were produced according to standard protocols ((Kohler and Milstein, 1975) as modified by Manser and Gefter (Manser and Gefter, 1984)). Hybridomas were cultured in RPMI-1640 (Whittaker Bioproducts, Walkersville, Maryland) supplemented with 10% Defined Fetal Calf Serum (Hyclone Laboratories, Logan, Utah) and either hypoxanthine, aminopterin, and thymidine (Sigma Chemical Company, St. Louis, Missouri) for HAT media, hypoxanthine and thymidine for HT media, or none of these for standard media. The fusion products were plated by limiting dilution in HAT medium. Supernatants from the original fusion wells were screened for anti-yeast tubulin reactivity by western blotting to total yeast protein extracts. 13/2000 (4/13 IgG) TUB1C clones and 9/1500 (4/9 IgG) TUB2C clones were positive for tubulin. The IgG-positive clones were all subcloned once by limiting dilution in HAT media. Reactivity to tubulin was confirmed by immunofluorescence staining of yeast (data not shown). One of the TUB1C clones (A1BG7) and one of the TUB2C clones (B1BE2) were subcloned a further two times in HT medium and used for the studies described in this paper. A1BG7 secretes an IgG3 with a k light chain. B1BE2 secretes an IgG2a with a k light chain. Ascites fluid was produce from these hybridomas by Charles River Laboratories (Wilmington, MA). IgG was purified from ascites fluid using a ProteinA IgG purification kit (Pierce, Rockford, IL), and covalently coupled overnight to Affigel-10 beads (Biorad Laboratories, Richmond, CA) in 50 mM HEPES pH 6.9. From 8-16 mg of protein was coupled per ml of beads. The active sites on the beads were blocked by 1M ethanolamine (MCB, Cincinnati, OH) in 50mM Hepes buffer, pH 6.9. Antibodies coupled to beads, (henceforth referred to as A1BG7 beads or B1BE2 beads), were used immediately for
immunoprecipitations. All animal care was in compliance with institutional guidelines.

Yeast Protein Preparations

*S. cerevisiae* strain CKY93 (MATa; leu2-3,112, pep4::URA3; ura3-52) (kindly provided by Dr. Chris Kaiser, MIT) was grown to late log phase in synthetic complete media minus uracil. (Katz, *et al.*, 1990; Sherman, *et al.*, 1986), harvested by centrifugation, and washed once in ice-cold PME buffer (0.1M Pipes pH 6.9, 1mM MgSO₄, 2 mM EGTA). The cell pellet was resuspended in two times the pellet volume cold PME buffer with protease inhibitors (1 µg/ml each of leupeptin, phenylmethylsulfonyl fluoride (PMSF), aprotinin, tosyl phenylalanine chloromethyl ketone (TPCK), and pepstatin (Sigma Chemical)). The cell suspension was twice passed through a cold French Pressure apparatus (SLM Aminco, Urbana, IL), using a 20,000 PSI pressure cell with a 3/8" diameter piston at an internal pressure of 20,000 PSI. The resultant cell lysate was cleared by centrifugation at 20,000g for 15 minutes at 4°C. The pellet was discarded and two times the supernatant volume cold PM3G (0.1M Pipes pH 6.9, 1mM MgSO₄, 2 mM EGTA, 3M Glycerol) with protease inhibitors was added to the cell extract.

Immunoprecipitations and Elutions

The freshly prepared yeast soluble protein extract was added in sufficient volume to saturate the A1BG7 or B1BE2 antibody coupled to beads (Chapter Four) and incubated for 4 hours, 4°C, on a tube rotator. The beads were washed five times with a minimum of 10 bead volumes cold PM2G (0.1M Pipes pH 6.9, 1mM MgSO₄, 2 mM EGTA, 2M Glycerol) and protease inhibitors and washed five times with a minimum 10 bead volumes cold PME with protease inhibitors. Protein preparations were quantitated by western blotting (see below).

A1BG7 and B1BE2 beads containing immunoprecipitated alpha- and beta-tubulin were eluted with the non-ionic detergents 0.1% NP-40 (Nonidet P-40; BDH Chemicals Ltd., Poole, England)) or 0.1% Triton X-114 (Octylphenoxypolyethoxyethanol, Sigma Chemical Co) in PME buffer, the control PME buffer alone, or 0.1% non-ionic detergent in PME buffer with 1mM
GTP at a range of 0.2 to 1.0 ml per mg of antibody beads. Samples were incubated for 90 minutes, 4°C, on a tube rotator. The antibody beads were pelleted and the pellet and supernatant fractions were characterized using western blotting techniques (see below). In some experiments, the antibody beads were first eluted with 0.1% Triton X-114 or 0.1% NP-40 in PM2G or PME, followed by a second elution with 0.1% Triton X-114 or 0.1% NP-40 in PME. The non-ionic detergents NP-40 and Triton X-114 behaved identically in all experiments.

**Protein Quantitation, Western Blotting, and Silver staining**

Protein concentrations from eluates and yeast soluble protein extracts were quantitated using the spectrophotometric DC Protein Quantitation assay (BioRad) according to the manufacturer's instructions.

Eluates, yeast soluble protein extracts, and purified yeast tubulin (Barnes, *et al.*, 1992) were diluted with 1/2 volume GSD (33% glycerol, 6.6% SDS, 0.35 M dithiothreitol), and boiled five minutes. All protein samples were loaded and run on 7.5% acrylamide Mini-Protean gels, and transferred to 0.2 μm nitrocellulose in a Mini-Protean gel transfer apparatus (BioRad) according to the manufacturer's instructions. Polyclonal anti-α-tubulin antisera, 345-4, and polyclonal anti-β-tubulin antisera, 206-1 (Bond, *et al.*, 1986; Schatz, *et al.*, 1988), and ¹²⁵I-Protein A (Dupont - NEN, Boston, MA) were used in western blots, as previously described (Fridovich-Keil, *et al.*, 1987). Blots were exposed to a phosphoimager screen overnight, scanned, and bands were quantitated using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Data was transferred to and manipulated on the spreadsheet program Excel (Microsoft, Redmond, WA). To compare signals from 345 and 206 western blots, values were normalized to signals from whole cell extracts.

7.5% Poly-acrylamide gels were silver-stained according to the protocol (Morrisey, 1981).
**GTP Binding**

GTP binding by photo cross-linking was adapted from conditions used in other systems (Penefsky, 1977; Yue and Schimmel, 1977). In experiments testing GTP binding to eluates, 6 µl of eluates from A1BG7 beads and 6 µl of eluates from B1BE2 beads were either added to each other, or to 6 µl of 0.1% Triton X-114 in PME with protease inhibitors. For other experiments, yeast whole cell extracts (see above) or assembly-competent purified yeast tubulin (Barnes, *et al.*, 1992) were diluted in 0.1% Triton X-114 in PME with protease inhibitors to tubulin concentrations similar to antibody eluates. The samples were brought to 15 µl with 1X MES buffer (40 mM MES, pH 6.4, 0.4 mM EGTA, and 0.2 mM MgCl₂) and incubated for 30 minutes at 4°C on a tube rotator. The protein samples were transferred to a 96-well plate flat bottom plate (Costar, Cambridge, MA). 5 µl of Hot GTP mix (2X MES, 0.16 mM ATP, 3 x 10⁻⁷ M³²P-α-GTP(Dupont-NEN), 4 µM GTP) were added to each well and the samples incubated on ice for 30 minutes. The proteins were mixed 1:1 with loading buffer, run immediately on one-dimensional gels, and transferred to nitrocellulose. Protein was visualized with 345-4 and 206-1 antisera (Bond, *et al.*, 1986; Schatz, *et al.*, 1988), and secondary antisera Alkaline-Phosphatase conjugated goat anti-rabbit (AP-GAR) according to the manufacturer's instructions (Promega). ³²P-α-GTP was visualized by exposure of blots to Kodak X-Omat film at -70°C with a screen or to a phosphoimager screen. Bands were quantitated using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Data was transferred to and manipulated on the spreadsheet program Excel (Microsoft, Redmond, WA). Arbitrary units were assigned to western and ³²P-α-GTP signals to allow direct comparisons.

In seven GTP binding experiments, the sum of the GTP binding signals for A1BG7 and B1BE2 eluates was always lower than the GTP binding signal in mixed eluates. Using the nonparametric statistical sign test, we determined that the probability of this occurring randomly was less than 0.02%. In contrast, using the same probability analysis, we determined that the sum of alpha- and beta-tubulin protein values from A1BG7 and B1BE2 eluates were not different from those in the mixed eluates (Randles and Wolfe, 1991).
Chapter Three

Results

We isolated α- and β-tubulin from *S. cerevisiae* whole cell extracts by immunoprecipitating the tubulin heterodimer using antibodies specific to the α- or β-tubulin chains. Monoclonal antibodies A1BG7 and B1BE2, generated against carboxy-terminal peptides of the major α-tubulin (TUB1) and the sole β-tubulin (TUB2) of *S. cerevisiae*, respectively, each recognized a single major band of ~55kD and stained yeast microtubules by indirect immunofluorescence (data not shown). We coupled A1BG7 or B1BE2 antibody to Affi-gel 10 beads (Materials and Methods) and incubated with freshly prepared yeast soluble protein extracts in PM2G, containing sufficient amounts of tubulin to saturate the antibody, for 4 hours at 4°C (Materials and Methods). A1BG7 antibody beads immunoprecipitated approximately equimolar amounts of α- and β-tubulin, as assayed by western blots stained with polyclonal antisera 345 and 206, which recognize yeast α- and β-tubulin, respectively (Figure 3.1). B1BE2 antibody also immunoprecipitated both β- and α-tubulin in approximately equimolar amounts, as assayed by western blotting (Figure 3.1). The control, Affi-gel 10 beads without antibody, failed to immunoprecipitate either tubulin chain (data not shown).

We disrupted the interaction between immunoprecipitated α- and β-tubulin by exposing the antibody:heterodimer complex to non-ionic detergents (Materials and Methods). Incubation of the antibody:heterodimer complex with 0.1% Triton X-114 in PME buffer for 90 minutes at 4°C eluted the tubulin chain not specifically recognized by the A1BG7 or B1BE2 antibody. Incubation of anti-α-tubulin antibody A1BG7 beads eluted 25% of the immunoprecipitated β-tubulin (Figure 3.2A). Incubation of anti-β-tubulin antibody B1BE2 beads under the same conditions eluted 27% of the immunoprecipitated α-tubulin (Figure 3.2B). There was some contamination by the antibody-specific tubulin chain in the detergent-mediated elutions, with more present in eluates from B1BE2 beads. Eluates from A1BG7 beads contained 3% of the immunoprecipitated α-
Figure 3.1. Immunoprecipitation of tubulin heterodimer with A1BG7 and B1BE2 monoclonal antibodies. Anti-α tubulin antibody, A1BG7 (lane 1), or anti-β-tubulin antibody, B1BE2 (lane 2), coupled to beads, were incubated with whole cell extracts from *S. cerevisiae*. The tubulin in pellet fractions was visualized using western blotting with anti-α-tubulin antiserum 345 or anti-β-tubulin antiserum 206, and secondary antibody ^125^I-Protein A (Materials and Methods). A1BG7 antibodies and B1BE2 antibodies immunoprecipitated approximately equal amounts of both α- and β-tubulin proteins.
A.

Figure 3.2. Buffer-dependent elution of tubulin from A1BG7 or B1BE2 beads. Anti-α-tubulin antibody A1BG7 (A) or anti-β-tubulin antibody B1BE2 (B) beads containing immunoprecipitated α- and β-tubulin were exposed to different buffer conditions to assess the efficiency of elution of α- and β-tubulin. Values, obtained from quantitative western blots stained with anti-α-tubulin antiserum, 345, and with anti-β-tubulin antiserum, 206 (Materials and Methods), were expressed as a percentage of immunoprecipitated tubulin. 0.1% Triton X-114 in PME buffer eluted ~25% of the β-tubulin immunoprecipitated by A1BG7 beads and ~27% of α-tubulin immunoprecipitated by B1BE2 beads. Only small amounts of the antibody-specific chain, ~3% of α-tubulin for A1BG7 beads and ~7% of β-tubulin for B1BE2 beads, were eluted under these conditions. Addition of 1mM GTP to 0.1% Triton X-114 in PME buffer inhibited the detergent-mediated separation of tubulin heterodimer, eluting both tubulin chains similarly to the control, PME buffer alone.
Figure 3.2 Buffer-dependent elution of tubulin from A1BG7 or B1BE2 beads. Anti-α-tubulin antibody A1BG7 (A) or anti-β-tubulin antibody B1BE2 (B) beads containing immunoprecipitated α- and β-tubulin were exposed to different buffer conditions to assess the efficiency of elution of α- and β-tubulin. Values, obtained from quantitative western blots stained with anti-α-tubulin antiserum, 345, and with anti-β-tubulin antiserum, 206 (Materials and Methods), were expressed as a percentage of immunoprecipitated tubulin. 0.1% Triton X-114 in PME buffer eluted ~25% of the β-tubulin immunoprecipitated by A1BG7 beads and ~27% of α-tubulin immunoprecipitated by B1BE2 beads. Only small amounts of the antibody-specific chain, ~3% of α-tubulin for A1BG7 beads and ~7% of β-tubulin for B1BE2 beads, were eluted under these conditions. Addition of 1mM GTP to 0.1% Triton X-114 in PME buffer inhibited the detergent-mediated separation of tubulin heterodimer, eluting both tubulin chains similarly to the control, PME buffer alone.
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Tubulin and eluates from B1BE2 beads contained 7% of the immunoprecipitated β-tubulin (Figure 3.2). However, the eluates from B1BE2 and A1BG7 beads were enriched for α- or β-tubulin, respectively, in the absence of its heterodimeric partner.

In the absence of 0.1% Triton X-114, the interaction between α- and β-tubulin heterodimer immunoprecipitated by A1BG7 or B1BE2 antibody beads was stable. Repeated washing with PME buffer failed to elute the chain not specifically recognized by the antibody. Incubation of the antibody:heterodimer complex with PME buffer for 90 minutes at 4°C, also failed to elute the non-specific chain (Figure 3.2). These results are consistent with the previously reported dissociation constant for the tubulin heterodimer in other organisms (Detrich III and Jr., 1978).

The elution of α- or β-tubulin was inhibited by the addition of GTP to the elution buffer containing non-ionic detergent. Incubation of the antibody:heterodimer complex with 0.1% Triton X-114 containing 1 mM GTP reduced the elution of α-tubulin from B1BE2 beads and β-tubulin from A1BG7 beads from 25% of the immunoprecipitated tubulin to near PME buffer control levels (Figure 3.2). These results suggest the tubulin heterodimer is stabilized by GTP under the conditions of assay, preventing the detergent-mediated separation of the tubulin heterodimer.

A second exposure of anti-tubulin antibody beads to 0.1% Triton X-114 in PME for 90 minutes at 4°C yielded an additional 25% of the immunoprecipitated β-tubulin from A1BG7 beads and 25% of the immunoprecipitated α-tubulin from B1BE2 beads, with generally lower quantities of the antibody-specific tubulin chain (Figure 3.3A). Since immunoprecipitated tubulin behaves identically in two consecutive elutions, it suggests the non-ionic detergent is not mediating separation of the tubulin chains by affecting an aberrant portion of the total immunoprecipitated pool.

The detergent-mediated elutions were highly enriched for tubulin relative to other yeast proteins. Eluates from both A1BG7 and B1BE2 beads contained a protein band that comigrated with tubulin from calf brain microtubule preparations on coomassie and silver-stained 7.5% polyacrylamide gels.
This band appeared to be tubulin by several criteria. First, it was not present in the eluates from control experiments, containing Affi-gel 10 beads but no antibody. Second, adding GTP to the Triton X-114 elution buffer or changing the concentration of Triton X-114 used in elution buffer decreased the intensity of the purported tubulin signal on silver-stained gels, consistent with the changes in the intensity of the tubulin signal as assessed by western blotting (see Chapter Four). The presence of a dominant tubulin protein signal on silver-stained gels represented a dramatic enrichment in the tubulin signal compared to the other proteins in the starting material (see Chapter Four).

The eluates from A1BG7 or B1BE2 beads were not entirely free of other protein bands. Since most of the other protein bands were present in control eluates, containing affi-gel 10 beads without antibody (see Chapter Four), they were likely due to the low stringency conditions of the immunoprecipitations. The non-specific bands were almost entirely eliminated from eluates by incubating A1BG7 or B1BE2 beads a second time in 0.1% Triton X-114 in PME or PM2G, for 90 minutes at 4°C (Figure 3.3B). The eluate preparations from a second elution of A1BG7 beads or B1BE2 beads were highly enriched for β- or α-tubulin, respectively, as assayed by western blots (Figure 3.3A). We calculated a greater than 500-fold enrichment for tubulin in the second elution relative to the starting material, based on spectrophotometric protein quantitation and relative western signals of yeast soluble protein extracts and eluates (See Chapter Four). From these values, and from estimates of tubulin protein concentrations in yeast soluble protein extracts (Barnes, et al., 1992), we calculated the purity of tubulin from the second eluate of A1BG7 or B1BE2 beads to be greater than 90%.

We determined the quantity of tubulin in the Triton X-114 eluate fractions by two methods. We compared tubulin bands of eluates from A1BG7 or B1BE2 beads to known quantities of tubulin from calf brain microtubule preparations (Kindly provided by Margaret Magendantz, M.I.T. (Sloboda and Rosenbaum, 1975)) on silver-stained and coomassie-stained gels. We also compared the tubulin signal of eluates from A1BG7 or B1BE2 beads with known quantities of purified yeast tubulin (Barnes, et al., 1992) on western blots, stained with polyclonal antisera 345 or 206. Both methods yielded similar results of approximately 15-20 μg/ml, or ~3 x 10^-7 M α- or β-tubulin (Chapter Four).
Figure 3.3 Tubulin is the predominant protein in eluates from A1BG7 or B1BE2 beads. (A) Western blots or (B) Silver-stained 7.5% polyacrylamide gels of elutions from anti-\(\alpha\)-tubulin antibody A1BG7 (lanes 1,2) or anti-\(\beta\)-tubulin antibody B1BE2 beads (lanes 3,4) with 0.1% Triton X-114 buffer. Anti-tubulin beads containing immunoprecipitated tubulin heterodimer were eluted once (lanes 1, 3) with 0.1% Triton X-114 in PME or twice (lanes 2, 4) with 0.1% Triton X-114 in PM2G followed by 0.1% Triton X-114 in PME. Western blots (A), visualized with anti-\(\alpha\)-tubulin antiserum 345, or anti-\(\beta\)-tubulin anti-sera 206 (Materials and Methods), demonstrated enrichment in eluates of the tubulin chain not specifically recognized by the antibody.
Figure 3.3  Tubulin is the predominant protein in eluates from A1E 7 or B1BE2 beads. (A) Western blots or (B) Silver-stained 7.5% polyacrylamide gels of elutions from anti-α-tubulin antibody A1BG7 (lanes 1,2) or anti-β-tubulin antibody B1BE2 beads (lanes 3,4) with 0.1% Triton X-114 buffer. Anti-tubulin beads containing immunoprecipitated tubulin heterodimer were eluted once (lanes 1, 3) with 0.1% Triton X-114 in PME or twice (lanes 2, 4) with 0.1% Triton X-114 in PM2G followed by 0.1% Triton X-114 in PME. On silver-stained gels (B), the arrow indicates migration of tubulin, as assessed from parallel lanes containing calf brain microtubule preparations (Kindly provided by Margaret Magendantz, M.I.T.(Sloboda and Rosenbaum, 1975)). Tubulin was the major protein in eluates from anti-α-tubulin antibody A1BG7, anti-β-tubulin antibody B1BE2 beads, but not control beads. These preparations were relatively pure with respect to other proteins.
The dissociation constant for tubulin under similar conditions (pH 7.0, no GTP, 10°C), but in the absence of detergent, is $\sim 1 \times 10^{-7}$ M as determined by the monomer-dimer distribution in sedimentation gradient coefficients (Shearwin, et al., 1994a). These results demonstrated that the tubulin prepared by detergent-mediated separation of the tubulin heterodimer was of sufficient purity and concentration to perform biochemical analysis of these polypeptides.

GTP can be covalently cross-linked to GTP-binding proteins by exposure to UV light (Penefsky, 1977; Yue and Schimmel, 1977). We used a modification of the photo-crosslinking procedures used for tubulin in other systems (Geahlen and Haley, 1977; Hesse, et al., 1987; Maruta, et al., 1986) to examine GTP binding to tubulin in S. cerevisiae (Materials and Methods). Briefly, yeast whole cell extracts, purified assembly-competent yeast tubulin (Barnes, et al., 1992), or eluates prepared by detergent mediated separation of tubulin from A1BG7 or B1BE2 beads, were incubated with $^{32}$P-$\alpha$-GTP in MES buffer on ice for 30 minutes. For experiments measuring the tubulin GTP dissociation constant, we varied the final concentration of GTP. In other experiments, the final GTP concentration was held constant at 1μM. We exposed the samples to UV light to covalently cross-link the GTP molecule to tubulin and ran these protein preparations on 7.5% polyacrylamide gels. The resultant western blots were stained with 345 or 206 polyclonal antisera, an alkaline phosphatase-conjugated secondary antibody to detect tubulin, and exposed to a phosphoimager screen or to film to quantitate the GTP signal that comigrated with the tubulin bands. To quantitate tubulin protein values, we examined identical but un-crosslinked preparations for tubulin using western blots, probed with $^{125}$I-Protein A secondary antibody, and exposed to a phosphoimager screen (Materials and Methods).

In cross-linked samples from yeast whole cell extracts, we identified $^{32}$P-$\alpha$-GTP signal that comigrated with tubulin. As reported in other systems (Geahlen and Haley, 1977; Hesse, et al., 1987; Maruta, et al., 1986), covalently cross-linked GTP remained associated with the β-tubulin subunit after separation of the tubulin heterodimer on two-dimensional gels (data not shown, L. Connell), further supporting the supposition that the GTP signal was due to tubulin GTP binding activity. We calculated the tubulin GTP dissociation constant for S.
cerevisiae from whole cell extracts to be $3 \times 10^{-7}$ M (data not shown, L. Connell), which is consistent with the tubulin GTP dissociation constant reported for other systems (Arai, et al., 1975).

We tested $\alpha$- or $\beta$-tubulin from S. cerevisiae, prepared by detergent-mediated separation of the tubulin heterodimer, for its ability to bind 1.0 $\mu$M GTP. A single $^{32}$P-$\alpha$-GTP band was present on western blots of these preparations, demonstrating that the major GTP binding activity co-migrated with tubulin (Chapter Four). We determined that the $^{32}$P-$\alpha$-GTP binding activity was specifically associated with beta-tubulin (data not shown). The results of seven GTP binding experiments are shown in Figure 3.4. GTP binding was enhanced when $\alpha$- and $\beta$-tubulin were both present, in equimolar concentrations. The same concentration of $\beta$-tubulin bound more GTP when equimolar concentrations of $\alpha$-tubulin were present than when $\alpha$-tubulin was limiting. The same concentration of $\alpha$-tubulin bound more GTP when equimolar concentrations of $\beta$-tubulin were present than when $\beta$-tubulin was limiting. The combined activity of A1BG7 or B1BE2 eluates alone was insufficient to explain the increase in GTP binding activity when the two eluates were mixed together (Materials and Methods). These results suggested that it was the level of potential heterodimer, not the level of $\alpha$- or $\beta$-tubulin, that correlated with GTP binding.

These data do not address whether monomeric or heterodimeric $\alpha$- and $\beta$-tubulin bind GTP, since GTP binding was not entirely absent from eluates containing predominantly $\beta$- or $\alpha$-tubulin. However, since the binding activity was reduced in preparations containing $\alpha$- or $\beta$-tubulin alone, each tubulin subunit must bind GTP less efficiently than the two subunits together. Alternatively, both tubulin subunits may be required for GTP binding activity, and the GTP binding activity in eluates enriched for $\alpha$- or $\beta$-tubulin is due to the presence of cross-contaminating tubulin polypeptides.
Figure 3.4 To assess GTP binding, eluates from anti-α-tubulin antibody A1BG7 and anti-β-tubulin antibody B1BE2 beads were mixed together or mixed with 0.1% Triton X-114 Buffer and incubated with $^{32}$P-α-GTP. Samples were UV cross-linked, and $^{32}$P-α-GTP binding to tubulin was quantified. Tubulin protein quantities for identical but uncross-linked fractions were determined from western blots stained with anti-α-tubulin antibody 345 or anti-β-tubulin antibody 206 (Materials and Methods). In order to compare GTP and tubulin signals from separate experiments, western and GTP signals from all three fractions were normalized to the signals in mixed eluates. The values shown represent the results of seven experiments. GTP binding is significantly enriched in mixed eluates relative to A1BG7 or B1BE2 bead eluates.
We compared the GTP binding activity of native tubulin heterodimer (Barnes, et al., 1992) with the activity of α- and β-tubulin prepared from the detergent-mediated separation of tubulin heterodimer. The single GTP binding activity present in purified tubulin heterodimer preparations comigrated with the tubulin signal on western blots and was linear over the range of tubulin concentrations tested (see Chapter Four). Recombined α- and β-tubulin from A1BG7 and B1BE2 eluates bound approximately the same amount of GTP as equal concentrations of α- and β-tubulin from purified tubulin heterodimer preparations (Figure 3.5). These data demonstrate that the GTP binding activity of native tubulin could be reconstituted in α- and β-tubulin prepared by detergent-mediated separation of the tubulin heterodimer.
Figure 3.5. $^{32}$P-α-GTP vs. Western Signals for Mixed Eluates and Purified Yeast Tubulin. The $^{32}$P-α-GTP binding activity was determined for purified yeast assembly-competent tubulin (Barnes, et al., 1992) and α- and β-tubulin produced from detergent mediated separation of tubulin heterodimer. Samples were incubated with $^{32}$P-α-GTP, UV cross-linked, run on western blots, and the $^{32}$P-α-GTP signal associated with tubulin protein quantified. Independent western blots stained with anti-α-tubulin antiserum 345 and anti-β-tubulin antiserum 206 were used to quantify protein values (Materials and Methods). Values shown are normalized to the signal in yeast soluble extracts to allow comparison of western and $^{32}$P-α-GTP values. $^{32}$P-α-GTP binding to yeast-assembly competent tubulin was linear across an eight-fold range of tubulin concentrations. Recombined α- and β-tubulin, produced from the detergent-mediated separation of tubulin heterodimer, bound similar quantities of $^{32}$P-α-GTP as equimolar concentrations of α- and β-tubulin from yeast purified assembly-competent tubulin. Note: The error bar on the 345 signal for Mixed eluates is nearly undetectable on this graph.
Discussion

We have described a procedure to isolate predominantly $\alpha$- or $\beta$-tubulin. Briefly, antibodies which specifically recognized either $\beta$- or $\alpha$-tubulin stably immunoprecipitated tubulin heterodimer from *S. cerevisiae* whole cells extracts. Exposure of the tubulin heterodimer to non-ionic detergent, which had been shown previously to subtly alter heterodimeric structure (Andreu, 1982; Andreu, 1986a; Andreu, *et al.*, 1989; Andreu and Munoz, 1986b; Andreu, *et al.*, 1986c) eluted the tubulin chain not specifically recognized by the antibody. Non-ionic detergents may destabilize the hydrophobic interface between $\alpha$- and $\beta$-tubulin, thought to be a critical component of the tubulin heterodimer, and thereby alter the tubulin dissociation constant. Consistent with this hypothesis, GTP, known to stabilize the tubulin heterodimer under these conditions (Shearwin, *et al.*, 1994a), inhibited the detergent-mediated separation of $\alpha$- and $\beta$-tubulin. Tubulin prepared by this procedure was relatively uncontaminated by other proteins, and present in sufficient concentrations to perform biochemical experiments.

Previous experiments have shown that $\beta$-tubulin is a component of tubulin GTP binding (Shearwin, *et al.*, 1994a). We tested $\alpha$- and $\beta$-tubulin prepared from eluates of A1BG7 and B1BE2 beads to determine whether $\alpha$-tubulin is also a necessary component of the tubulin GTP binding activity. The results of a GTP UV-cross-linking assay demonstrated that $\beta$-tubulin alone and $\alpha$-tubulin alone were insufficient to reconstitute the GTP binding activity observed in assembly-competent native tubulin heterodimer. However, when $\alpha$- and $\beta$-tubulin were mixed together, they bound GTP like native tubulin heterodimer by several criteria. These molecules cross-linked GTP identically to native tubulin, tested from whole cell extracts or purified, assembly-competent tubulin heterodimer. The GTP concentration, 1.0 $\mu$M, was slightly above the GTP tubulin dissociation constant in yeast, 0.3 $\mu$M. Finally, tubulin produced from eluates of A1BG7 and B1BE2 beads bound the same number of GTP molecules as an equal concentration of tubulin heterodimer from yeast purified assembly-competent tubulin preparations.
These results suggest both tubulin subunits, not α- or β-tubulin alone, are required for GTP binding, consistent with a previously reported *in vitro* GTP binding assay (Farr, *et al.*, 1990). Other results, such as photo cross-linking experiments or mutations in β-tubulin that alter GTP binding and hydrolysis (Shearwin, *et al.*, 1994a), do not preclude this interpretation since in each of these cases GTP binding was assayed in tubulin heterodimers. GTP binding may occur at the α–β-tubulin interface, which could explain the absence of a typical GTP binding and hydrolysis consensus sequence in β-tubulin alone (Dever, *et al.*, 1987; Sternlicht, *et al.*, 1987). Alternatively, GTP binding may occur in β-tubulin, but the presence of α-tubulin is required for β-tubulin to acquire or maintain an appropriate GTP binding conformation.
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Chapter Four

The Detergent-Mediated Separation of α- and β-tubulin: Experimental Parameters
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Introduction

The procedure to separate alpha- and beta-tubulin arose from an oddity in a control lane of an immunoprecipitation experiment, performed by Brant Weinstein while he was a graduate student in the Solomon laboratory (Weinstein, 1992). Both alpha- and beta-tubulin polypeptides were immunoprecipitated by polyclonal antibodies generated against either chain, consistent with previous reports in the literature. However, antibodies generated against alpha- or beta-tubulin preferentially immunoprecipitated the tubulin chain specific for the antibody in identical buffers containing low concentrations of the non-ionic detergent Nonidet P-40. These data suggested that alpha- and beta-tubulin polypeptides were dissolvable from one another by non-ionic detergents.

A number of experiments were required to transform the observation that non-ionic detergents mediate the separation of alpha- and beta-tubulin into a reproducible procedure. In addition, a number of parameters were tested to refine the procedure for specific experimental purposes. These experiments are described in this chapter.
Materials and Methods

Strains, Media, and Culture Conditions

Saccharomyces cerevisiae strains used in this study were CKY93, (MATa; leu2-3,112, pep4::URA3; ura3-52), FSY185 (ura3-52/ura3-52, leu2-3,112/leu2-3,112, lys801, his3-Δ200/his3-Δ200, TUB2/TUB2), and FSY182 (ura3-52, leu2-3,112/leu2-3,112, lys801/lys801, his3-Δ200/his3-Δ200, Tub1::HIS3, tub3:TRP1, TUB1p, LEU2p). CKY93 contains a disruption of the PEP4 locus, which reduced endogenous protease activity (kindly provided by Chris Kaiser). The strains were grown in synthetic complete media minus amino-acids specific for the strain auxotrophic markers (Katz, et al., 1990; Sherman, et al., 1986). Hybridomas were cultured in RPMI-1640 (Whittaker Bioproducts, Walkersville, Maryland) supplemented with 10% Defined Fetal Calf Serum (Hyclone Laboratories, Logan, Utah) and either hypoxanthine, aminopterin, and thymidine (all obtained from Sigma Chemical Company, St. Louis, Missouri) for HAT media, hypoxanthine and thymidine for HT media, or none of these for standard media.

Monoclonal Antibody Generation

The two peptides used to generate monoclonal antibodies were NH2-CADSYAEEEE-COOH (TUB1C peptide; Biopolymers Laboratory, Center for Cancer Research, M. I. T.) and NH2-CQNQDEPITENFE-COOH (TUB2C peptide; Clontech, Palo Alto, California). The peptides were coupled to Keyhole Limpet Hemocyanin (KLH, Sigma Chemical) using standard methods (Harlow and Lane, 1988). Four 6-week old Balb/C mice were injected intraperitoneally with approximately 50 mg of KLH-conjugated TUB1C peptide emulsified in CFA. Another four 6-week old Balb/C mice were injected with approximately 50 mg of KLH-conjugated TUB2C peptide emulsified in CFA. Both sets of mice were boosted at approximately 3 week intervals with 50 mg of the appropriate KLH-conjugated peptide in Freund's incomplete adjuvant. The appearance of anti-tubulin antibodies in the serum of the mice was monitored by western blotting of tail bleeds. Both sets of mice were boosted a total of 6 times with KLH-conjugated peptide in IFA. Three days after the final boost the mice were killed and their spleens removed. The splenocytes were fused to P3X63-Ag8.653 cells.
to generate hybridomas, by the method of Kohler and Milstein (Kohler and Milstein, 1975) as modified by Manser and Gefter (Manser and Gefter, 1984). The fusion products were plated by limiting dilution in HAT medium. Supernatants from the original fusion wells were screened for anti-yeast tubulin reactivity by western blotting to total yeast protein extracts. 13/≈2000 (4/13 IgG) TUB1C clones and 9/≈1500 (4/9 IgG) TUB2C clones were positive for tubulin. The IgG-positive clones were all subcloned once by limiting dilution in HAT media. Reactivity to tubulin was confirmed by immunofluorescence staining of yeast. One of the TUB1C clones (A1BG7) and one of the TUB2C clones (B1BE2) were subcloned a further two times in HT medium and used for the studies described in this paper. A1BG7 secreted an IgG3 with a k light chain. B1BE2 secreted an IgG2a with a k light chain. Ascites fluid was produced from these hybridomas by Charles River Laboratories (Wilmington, MA). IgG was purified from ascites fluid using a ProteinA IgG purification kit (Pierce, Rockford, IL), and covalently coupled overnight to Affigel-10 beads (Biorad Laboratories, Richmond, CA) in 50 mM HEPES pH 6.9. 8-16 mg of protein were coupled per ml of beads. For controls, we incubated Affi-gel10 beads in buffer alone. After, incubation with antibodies or buffer from several hours to overnight, the active sites on the beads were blocked by a one hour incubation in 1 M ethanolamine (MCB, Cincinnati, OH) in 50mM Hepes buffer, pH 6.9. Antibodies coupled to beads, (henceforth referred to as A1BG7 beads, B1BE2 beads, or Blank beads), were used immediately for immunoprecipitations.

Preparation of yeast total soluble protein extracts

*S. cerevisiae* strains were grown to late log phase, harvested by centrifugation, and washed once in cold PME buffer (0.1M Pipes pH 6.9, 1mM MgSO4, 2 mM EGTA ). The cell pellet was resuspended in two times the pellet volume cold PME buffer with protease inhibitors (1 μg/ml each of leupeptin, phenylmethylsulfonyl fluoride (PMSF), aprotinin, tosyl phenylalanine chloromethyl ketone (TPCK), and pepstatin (all obtained from Sigma Chemical)). The cell suspension was twice passed through a cold French Pressure apparatus (SLM Aminco, Urbana, IL), using a 20,000 PSI pressure cell with a 3/8" diameter piston at an internal pressure of 20,000 PSI. The resultant cell lysate was cleared by centrifugation at 27,000g for 15 minutes at 4ºC. The pellet was discarded and, in
most experiments, the supernatant was diluted two-fold with cold PM3G (0.1M Pipes pH 6.9, 1mM MgSO₄, 2 mM EGTA, 3M Glycerol) with protease inhibitors was added to the cell extract. In experiments characterizing the immunoprecipitation buffer, the supernatant was diluted two-fold in cold PME with protease inhibitors with or without 1 mM GTP. The soluble protein extracts were used immediately for immunoprecipitations because freezing these preparations resulted in diminished activity. A fraction of these preparations were diluted with 1/2 volume GSD (33% glycerol, 6.6% SDS, 0.35 M dithiothreitol), and boiled five minutes, to determine, by western blotting (see below), the amount of alpha- and beta-tubulin present in the starting material.

**Immunoprecipitation of tubulin and isolation of tubulin monomers.**

In most experiments, yeast soluble protein extracts were added in sufficient volumes to saturate the anti-alpha tubulin or anti-beta-tubulin antibody coupled to beads and incubated on a tube rotator at 4°C for four hours. To determine the volume of yeast soluble extract required for maximal binding to and elution from anti-alpha- and anti-beta-tubulin beads, we varied the extract volume from 0.5 ml to 25 ml per 1 mg of A1BG7 or B1BE2 beads. To test the ideal length incubation time under variable buffer conditions, incubation time was varied from 30 minutes to 5 hours.

The beads containing immunoprecipitated tubulin were washed five times with a minimum of 10 bead volumes cold immunoprecipitation buffer, (PM2G or PME) with protease inhibitors and washed five times with a minimum 10 bead volumes cold PME with protease inhibitors. In some experiments, a portion of the immunoprecipitate was removed, diluted with 1/2 volume GSD (33% glycerol, 6.6% SDS, 0.35 M dithiothreitol), and boiled five minutes, to determine, by western blotting (see below), the amount of alpha- and beta-tubulin immunoprecipitated with the anti-tubulin antibodies.

To test for elution of the tubulin chains, tubulin immunoprecipitated by antibody beads was eluted by adding PME with protease inhibitors containing 0.1% Triton X-114 (Octylphenoxypolyethoxyethanol, Sigma Chemical Co, St. Louis, MO) or 0.1% NP-40 (Nonidet P-40; BDH Chemicals Ltd., Poole, England) and incubating on a tube rotator at 4°C or 90 minutes. The time required for optimal elution was determined by altering the elution times. The elution volume
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ranged from 0.15 ml to 2.5 ml per mg of antibody beads in order to determine the optimal amount and concentration of tubulin eluted. In experiments examining the role of GTP in monomer elution, 1mM GTP was added to the detergent/PME solution during the elution step. In experiments examining the role of glycerol in monomer elution, 2M glycerol was added to the detergent/PME solution during the elution step. Following incubation, the supernatant containing tubulin was carefully removed. It was either diluted with 1/2 volume GSD, and boiled 5 minutes, or used immediately for additional experiments. The pellet was resuspended in PME, and boiled with 1/2 volume GSD for 5 minutes.

**Protein Quantitation, Western Blotting, and Silver-staining**

Total protein concentrations in eluate fractions and yeast soluble extracts were determined using the BioRad DC Protein Assay according to the manufacturers instructions (BioRad). To quantitate tubulin, protein samples were loaded in duplicate or triplicate and run on 7.5% SDS-Polyacrylamide mini-protean gels, and transferred to 0.2μm nitrocellulose according to the manufacturers instructions (BioRad). Polyclonal anti-alpha-tubulin antisera, 345-4 (Schatz, *et al.*, 1988), and polyclonal anti-beta-tubulin antisera, 206-1 (Bond, *et al.*, 1986) were used in western blots, as previously described (Fridovich-Keil, *et al.*, 1987). Blots were exposed to a phosphoimager screen overnight, scanned, and bands were quantitated using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The data was transferred to the spreadsheet program Excel (Microsoft, Redmond, WA). In order to compare data from 345-4 and 206-1 western blots, all values for eluate and pellet fractions were normalized to the tubulin values in yeast whole cell extracts or in purified yeast tubulin preparations (kindly provided by Georjana Barnes (Barnes, *et al.*, 1992)).

To examine the purity of pellet and eluate fractions, samples were loaded and run on a 7.5% polyacrylamide gels and fixed and stained in 50% Methanol with 7.5% Acetic acid containing Coomassie blue stain. After destaining with 50% Methanol/7.5% Acetic acid, gels were silver-stained according to a standard protocol (Morrisey, 1981). Microtubule preparations from calf brains (MTP) (kindly provided by Margaret Magendantz, (Sloboda and Rosenbaum, 1975)) were used as an internal control for tubulin mobility and provided estimates of tubulin concentration.
Results

The basic procedure for the detergent-mediated separation of alpha- and beta-tubulin is outlined in Figure 4.1 and has been described in Chapter 3 of this dissertation. Briefly, A1BG7 and B1BE2 monoclonal antibodies, coupled to beads were incubated for four hours at 4°C with yeast soluble protein extracts in PM2G. After washing, the antibody-antigen complex was exposed to Pipes buffer with a variety of additional buffer components, including glycerol, non-ionic detergent, and GTP. Pellet and eluate fractions were examined by western blotting and silver-stained polyacrylamide gel analysis to characterize the immunoprecipitated components.

Under the conditions of the protocol, alpha- and beta-tubulin were immunoprecipitated in an approximate 1:1 molar ratio by A1BG7 or B1BE2 antibodies (See Figure 3.1, Chapter 3). Addition of 0.1% Triton X-114 in PME buffer to the immunoprecipitated tubulin eluted the tubulin chain not specifically recognized by the antibody. Western blots of the resultant eluates are shown in Figure 4.2. Eluates from A1BG7 beads were greatly enriched for beta-tubulin (Figure 4.2.A) and eluates from B1BE2 beads were greatly enriched for alpha-tubulin (Figure 4.2.B). The separation of the tubulin chain from heterodimer was due to the presence of the non-ionic detergent since A1BG7 or B1BE2 beads eluted in PME alone were not enriched for beta- or alpha-tubulin, respectively, as assessed by western blotting (Figure 4.2). The detergent-mediated separation of tubulin heterodimer was consistent with reports from the literature that detergents destabilized alpha-beta-tubulin interactions (Andreu, 1982; Andreu, 1986a; Andreu, et al., 1989; Andreu and Munoz, 1986b; Andreu, et al., 1986c; Sloboda and Rosenbaum, 1975). These data were examined quantitatively in Chapter 3 of this dissertation (see Figure 3.2).
Figure 4.1. The Experimental Protocol for the Detergent-Mediated Separation of Alpha- and Beta-tubulin. Anti-alpha-tubulin antibody A1BG7 or anti-beta-tubulin antibody B1BE2, coupled to affi-gel 10 beads, were incubated with yeast soluble extract in PM2G for 4 hours. The tubulin-chain not specifically recognized by the antibody was eluted from the antibody-antigen complex with a 90 minute incubation in buffer containing non-ionic detergent.
Figure 4.2. Western Blots of Elutions of Alpha- and Beta-tubulin from A1BG7 and B1BE2 Beads with Different Buffers. (A) A1BG7 or (B) B1BE2 beads were incubated with PME or 0.1% Triton X-114 in PME with or without 2M glycerol or 1mM GTP. The resultant eluates were examined by western blot analysis, as described in the Materials and Methods. Blots were stained with anti-alpha-tubulin antisera 345 or anti-beta-tubulin antisera 206.
Immunoprecipitation

The immunoprecipitation of alpha- and beta-tubulin was affected by a number of experimental parameters, including incubation times, buffer components, and protein preparations. These variables had effects on the immunoprecipitation of tubulin heterodimer and, in some cases, effects on the detergent-mediated separation of tubulin polypeptides. They are described below.

Preparation of Yeast Soluble Protein Extracts

We examined the detergent-mediated separation of alpha- and beta-tubulin in protein preparations from several *S. cerevisiae* strains, including FSY185, (wildtype diploid), FSY182 (haploid with ΔTUB3 and TUB1 on a plasmid), and CKY93 (ΔPEP4). Alpha- and beta-tubulin produced from each of these strains behaved identically in immunoprecipitation and elution protocols, as assayed by western blotting. We chose the CKY93 strain because it contained a deletion in the PEP4 locus, greatly reducing yeast endogenous protease activity. It was critical that the yeast soluble protein preparations used in immunoprecipitations be freshly prepared. Greater than 90% of the A1BG7 or B1BE2 binding activity was lost from yeast soluble protein extracts that had been flash frozen in liquid nitrogen and stored at -80°C.

Monoclonal Antibody Preparations

A1BG7 and B1BE2 antibodies were produced, purified and coupled according the procedures described in materials and methods. In the course of this work, we discovered that A1BG7 or B1BE2 antibodies coupled to Affi-gel10 beads lost most of their tubulin binding activity when they were frozen, stored for several days at -80°C, and thawed. Affi-gel10 coupled A1BG7 and B1BE2 antibody could be stored at 4°C, where they retained total binding activity for at least several days. In nearly all experiments, antibodies were coupled to beads immediately prior to use.
**Immunoprecipitation Buffer**

To determine whether the composition of the immunoprecipitation buffer affected the immunoprecipitation or elution of alpha- and beta-tubulin from A1BG7 or B1BE2 beads, we tested three immunoprecipitation buffers: PME alone, PME with 2M Glycerol, or PME with 1mM GTP. Immunoprecipitation of alpha- and beta-tubulin by A1BG7 or B1BE2 beads occurred efficiently with each of these buffers. However, the specific buffer used for the immunoprecipitation of tubulin by A1BG7 or B1BE2 beads had important consequences for the elution of alpha- and beta-tubulin from the antibody beads. The presence of GTP in the immunoprecipitation buffer reduced both the amount of beta-tubulin eluted from A1BG7 beads and the amount of alpha-tubulin eluted from B1BE2 beads (data not shown). These affects may have been due to low but sufficient concentrations of residual GTP contaminating the bead matrix. Alternatively, GTP could have long lasting effects on heterodimer stability, competing with the effects of non-ionic detergents.

Elution of alpha- and beta-tubulin from B1BE2 or A1BG7 beads, respectively, occurred efficiently with PME buffer, with or without 2M glycerol. However, tubulin prepared according to the conditions of this procedure has been shown by others to be less stable in the absence of guanine nucleotides (Shearwin, *et al.*, 1994a). Since tubulin was stabilized by 2M glycerol (Frigon and Timasheff, 1975; Solomon, *et al.*, 1973) we included 2M Glycerol (PM2G) in the immunoprecipitation buffers in the remaining experiments described in this appendix, unless noted.

**Time of Binding**

To maximize the amount of alpha- and beta-tubulin immunoprecipitated by A1BG7 or B1BE2 beads, we determined the ideal length incubation time. Equal volumes of yeast soluble extract were added to A1BG7 or B1BE2 beads and incubated from 0.5 to 4.5 hours. Immunoprecipitation pellets containing tubulin were examined by quantitative western blotting, as described in the materials and methods. Binding of alpha- and beta-tubulin in PM2G buffer for each antibody reached saturation by 3.5 to 4.0 hours of incubation. Immunoprecipitations from yeast soluble extracts in PME, with or without 10µM GTP, were far more rapid,
reaching saturation after 30 minutes of incubation. Clearly, the high viscosity of glycerol increased the incubation time in these experiments. In all other experiments described in this appendix, yeast soluble protein extracts in PM2G were incubated with A1BG7 or B1BE2 beads for 3.5 to 4.5 hours.

**Saturation of Antibody Binding Sites**

The total volume of yeast soluble extract required to saturate A1BG7 or B1BE2 antibodies was determined from two similar experimental approaches. First, aliquots containing constant amounts of A1BG7 or B1BE2 antibody were incubated with increasing volumes of yeast soluble extract in PM2G. Alternatively, we held the volume of yeast soluble extract constant and incubated with increasing concentrations of A1BG7 or B1BE2 antibody. In each case, the immunoprecipitated material was collected and analyzed by western blotting.

The results of each experimental approach yielded similar values for the volume of yeast soluble protein extract required to saturate a known quantity of A1BG7 or B1BE2 beads. The results of first approach are shown in Figure 4.3(A and B). ~5.0 ml of yeast soluble extract were required to saturate 0.5 mg of A1BG7 beads with alpha-tubulin. ~5.0 ml of yeast extract were required to saturate 0.5 mg of B1BE2 antibodies with beta-tubulin. Saturation binding of the tubulin chain not specifically recognized by the antibody occurred under identical parameters except that the total amount bound was consistently lower.

The results of these experiments allowed us to estimate the total amount of alpha- and beta-tubulin bound by 0.5 mg of A1BG7 or B1BE2 antibody coupled to beads. The total protein concentration in yeast soluble protein extracts was ~10 mg/ml (Table 1), of which ~0.1%, or ~10μg/ml, was tubulin (L. Pillus and F. Solomon, unpublished). Since half-maximal saturation occurred at ~ 2 ml of yeast soluble protein extract per 0.5 mg of beads, ~40μg of the antibody-specific tubulin chain were required to saturate the antibody beads. Theoretically, 500 μg of A1BG7 or B1BE2 antibody should bind 160 μg of the antibody-specific tubulin chain, if binding is 1 mol :1 mol and each antibody contains a single active site. These results demonstrated loss of ~75% of the immuno-reactive sites during antibody manipulations or coupling.
Figure 4.3. Immunoprecipitation and Elution of Alpha- and Beta-Tubulin by A1BG7 or B1BE2 Beads as a Function of Yeast Soluble Extract Volume. 0.5 mg of A1BG7 or B1BE2 antibody, coupled to beads, were incubated with increasing volumes of yeast soluble extracts in PM2G. (A) and (B) The amount of alpha- and beta-tubulin immunoprecipitated by A1BG7 (A) or B1BE2 (B) beads incubated with increasing volumes of yeast soluble extracts. Tubulin signals were quantified from western blots stained with anti-alpha-tubulin antisera 345 or anti-beta-tubulin antisera 206 (Materials and Methods). Values shown are relative to yeast soluble extract tubulin signals.
Figure 4.3. (continued) Amount of Alpha- and Beta-tubulin eluted from A1BG7 beads (C) or B1BE2 beads (D) containing tubulin immunoprecipitated from increasing volumes of yeast soluble extract. Tubulin signals were quantified from western blots stained with anti-alpha-tubulin antisera 345 or anti-beta-tubulin antisera 206 (Materials and Methods). Values shown are relative to immunoprecipitated tubulin.
We determined whether A1BG7 or B1BE2 antibodies saturated with alpha- or beta-tubulin, respectively, would elute the tubulin chain efficiently. In theory, empty antibody binding sites might capture antibody-specific tubulin chains and thereby reduce the contamination of antibody-specific chain in the eluates. Alternatively, saturated beads would contain larger amounts of tubulin available for elution, thereby increasing elution efficiency. We tested these alternatives by eluting from constant antibody weight beads exposed to increasing volumes of extract (Figure 4.3, A and B).

The results of this experiment are shown in Figure 4.3 (C and D). The elution efficiency, measured as the fraction of tubulin eluted over the amount immunoprecipitated, increased as the beads became more saturated, leveling off at just higher than half-maximal saturation. The relative amounts of alpha-present in eluates from A1BG7 were small under all conditions (Figure 4.3C). The amount of beta-tubulin present in eluates from B1BE2 beads, increased as the beads became saturated. However, the ratio of alpha-tubulin to beta-tubulin was not dramatically altered (Figure A.4D). Saturated beads did produce a larger pool of tubulin in the eluates since they immunoprecipitated larger amounts of tubulin. As a consequence, subsequent immunoprecipitations were performed under conditions where antibody beads were saturated.
Elution

The elution of alpha- and beta-tubulin from A1BG7 or B1BE2 beads was affected by a number of experimental parameters, including incubation times, buffer composition, and eluant volume. These variables altered the efficiency of elution and, in some cases, the concentration of alpha- and beta-tubulin present in the eluates. The results of these experiments provided a baseline from which to manipulate total tubulin eluted and the concentration of tubulin in the eluates, two important variables in functional tests of the monomeric alpha- and beta-tubulin. The experiments examining elution parameters are described below.

Buffer Composition

Effects of GTP and Glycerol

The precise composition of the eluant buffer proved critical for the efficient elution of alpha- and beta-tubulin from B1BE2 and A1BG7 beads. The addition of 1 mM GTP to the 0.1% Triton X-114 eluant buffer in PME reduced the amount of alpha- and beta-tubulin eluted from A1BG7 and B1BE2 beads to near PME buffer control levels, as examined by western blotting (Figure 4.2). A more extensive examination of this data is contained in Chapter Three.

Glycerol also inhibited the detergent-mediated separation of alpha- and beta-tubulin from anti-tubulin antibody beads. Addition of 2M glycerol to the 0.1% Triton X-114/PME eluant buffer reduced the total amount of alpha-tubulin or beta-tubulin eluted from B1BE2 or A1BG7 beads, respectively, based on western blotting analysis (Figure 4.2), although the reduction was not as dramatic as that caused by GTP.

The precise action of glycerol on the elution procedure was not entirely clear. Reports from the literature demonstrated that glycerol stabilized the tubulin heterodimer, decreasing the dissociation constant, as measured by sedimentation coefficient experiments (Detrich III, et al., 1982). However, Triton X-114 solubility was altered in buffers containing glycerol, causing the detergent to reach cloud point at temperatures near 40°C (Werck-Reichhart, et al., 1991). It was possible that either or both of these effects were responsible for the
inhibitory effects of glycerol on the elution of tubulin chains from anti-tubulin antibodies. We did not investigate further which, if either, of these factors was involved.

**Detergent Concentration**

We examined the effects of detergent concentration on the elution of alpha- and beta-tubulin from anti-tubulin antibody beads. Equal quantities of A1BG7 or B1BE2 beads were exposed to increasing concentrations of Triton X-114 detergent in PME buffer. To determine the amount of alpha- and beta-tubulin present and the purity of tubulin with respect to other proteins, the resultant eluates were examined by quantitative western blotting and silver-stained gel analysis.

The results of a western blotting experiment are shown in Figure 4.4. Generally, higher concentrations of Triton X-114 eluted beta-tubulin from A1BG7 beads with greater efficiency than low concentrations, consistent with the hypothesis that the separation of tubulin heterodimer is mediated by detergents. However, no increase in elution efficiency occurred at concentrations tested that were greater than 0.1% Triton X-114. All concentrations of Triton X-114 eluted alpha-tubulin from A1BG7 beads poorly (Figure 4.4.A).

The results from experiments with B1BE2 beads were similar (Figure 4.4 B). Generally, higher concentrations of Triton X-114 eluted alpha-tubulin from B1BE2 beads more effectively than lower concentrations. Again, elution efficiency did not increase when detergent concentrations were greater than 0.1% Triton X-114. With increasing detergent concentrations, the amount of beta-tubulin eluted from B1BE2 beads increased in parallel with the alpha-tubulin eluted, although the amount of alpha-tubulin was consistently higher. It is unclear why the presence of beta-tubulin in eluates from B1BE2 beads continues to increase since it is unlikely that these concentrations of detergent interfere with antibody:antigen interactions.
Figure 4.4. Elution of Alpha- and Beta-tubulin from A1BG7 and B1BE2 Beads vs. Triton X-114 concentration. Triton X-114 elutes beta-tubulin from A1BG7 (A) beads or (B) B1BE2 beads in a concentration-dependent manner. Antibody beads containing immunoprecipitated alpha- and beta-tubulin were eluted with increasing concentrations of Triton X-114 in PME. Values were obtained from quantitative western blots, stained with anti-alpha-tubulin antisera, 345, and with anti-beta-tubulin antisera, 206, and are shown relative to tubulin concentration in the starting material.
Figure 4.5. Silver stain of eluates from A1BG7 or B1BE2 Beads vs. Triton X-114 Concentration. Silver-stained 7.5% Acrylamide gels containing eluates produced from exposing A1BG7 or B1BE2 beads to increasing concentrations of Triton X-114 detergent in PME buffer. As a control, a microtubule preparation from calf brain, (MTP), which predominantly contains tubulin, is shown in the far left lane. The prominent band, with arrow, is tubulin. Detergent concentrations tested in these experiments were (lane 1) 0.5%; (lane 2) 0.1%; (lane 3) 0.01%; lane 4 (0.003%); (lane 5) 0.001%; (lane 6) 0% Triton X-114 in PME.
Eluates from A1BG7 or B1BE2 beads produced from increasing concentrations of detergent in the eluant were also examined by silver-stained gel analysis. The results are shown in Figure 4.5. The tubulin band was identified on these gels based on the migration of tubulin from calf-brain microtubule preparations in parallel lanes (kindly provided by Margaret Magendantz) and because the intensity of these bands increased with increasing detergent concentrations in a manner consistent with western blotting analysis. The intensity of at least some of the other bands in these lanes was of similar intensity regardless of the detergent concentration, suggesting they were non-specific.

**Volume of Eluant**

We examined the effects of altered eluant volumes on the elution of alpha- and beta-tubulin from A1BG7 and B1BE2 beads. Equal amounts of A1BG7 or B1BE2 beads, containing immunoprecipitated tubulin heterodimer were exposed to increasing volumes of 0.1% Triton X-114 in PME. The resultant eluates were examined by quantitative western blotting.

The results of this experiment are shown in Figure 4.6. The concentration of beta-tubulin eluted from A1BG7 beads and alpha-tubulin eluted from B1BE2 beads, expressed as a fraction of the western signal from yeast soluble protein extracts, decreased as the volume of eluant buffer increased (Figure 4.6.A and B). The concentration of antibody-specific tubulin chain eluted from these beads was far less sensitive to eluant volume.

While smaller eluant volumes increased the concentration of tubulin in the eluates, larger eluant volumes increased the total amount of alpha- and beta-tubulin eluted from B1BE2 and A1BG7 beads, respectively. These results are shown in Figure 4.6, C and D. The fraction of immunoprecipitated beta-tubulin eluted from A1BG7 beads initially increased as the volume of eluant increased, although it saturated at approximately 30%. The amount of alpha-tubulin eluted from these beads remained low. The fraction of immunoprecipitated alpha-tubulin eluted from B1BE2 beads also increased as eluant volume increased, to greater than 40% of the immunoprecipitated tubulin. The fraction of beta-tubulin present in these eluates increased in a similar manner.
Figure 4.6. Concentration and Quantity of Tubulin from Eluates of A1BG7 or B1BE2 Beads vs. Elution Volume. 0.6 mg A1BG7 beads (A) or 0.6 mg B1BE2 beads (B), pre-incubated with yeast soluble extract, were split into equal fractions, and each fractions was exposed to different volumes of 0.1% Triton X-114 in PME. The concentrations of eluates (A and B) were determined by quantitative western blots, using 345 and 206 antisera which recognize Tub1p and Tub2p respectively, and are expressed here as a fraction of tubulin concentration from yeast soluble extracts.
Figure 4.6. Concentration and Quantity of Tubulin from Eluates of A1BG7 or B1BE2 beads vs. Elution Volume (continued). 0.6 mg A1BG7 beads (C) or 0.6 mg B1BE2 beads (D), pre-incubated with yeast soluble extract, were split into equal fractions, and each fractions was exposed to different volumes of 0.1% Triton X-114 in PME. (C and D) The amount of tubulin eluted, obtained from western blotting, is expressed as a fraction of the tubulin immunoprecipitated by A1BG7 beads.
The results of experiments examining eluant volume were quite useful because they provided a mechanism by which the amount and concentration of alpha- and beta-tubulin present in the eluates could be manipulated. Generally, we chose to keep eluant volumes low to increase the concentration of tubulin in the eluates.

**Elution Incubation Time**

We examined the effects of incubation time on the detergent-mediated separation of alpha- and beta-tubulin heterodimer from anti-tubulin antibody. A1BG7 or B1BE2 beads, preincubated with yeast soluble extracts, were incubated in a constant volume 0.1% Triton X-114 for 15 to 180 minutes. The resultant eluates were examined by western blotting analysis.

The results, shown in Figure 4.7(A), demonstrated that the elution of beta-tubulin from A1BG7 beads occurred rapidly. Approximately 12% of the immunoprecipitated beta-tubulin eluted in the first 15 minutes. The fraction of immunoprecipitated beta-tubulin in these eluates continued to increase, to greater than 22% over 180 minutes of incubation. The presence of the antibody-specific chain, alpha-tubulin, remained low in these preparations.

The percentage of alpha-tubulin eluted from B1BE2 beads did not rise as quickly as that from A1BG7 beads. However, the fraction of alpha-tubulin eluted from B1BE2 beads was nearly twice that of the beta-tubulin eluted from A1BG7 beads, reaching ~40% of the immunoprecipitated tubulin. The amount of beta-tubulin in these fractions remained near or below 10%.

We chose 90 minute incubation times for most elution experiments. Elution from A1BG7 beads was nearly as high with 90 minute incubations as with 180 minute incubations. Elutions from B1BE2 beads with 90 minute incubations contained nearly 30% of the immunoprecipitated tubulin. Longer incubations increased the amount of tubulin eluted from A1BG7 and B1BE2 beads, but we were concerned that tubulin produced from longer incubations might be more susceptible to denaturation since glycerol and GTP were absent from these eluant buffers.
Figure 4.7. Alpha and Beta-Tubulin Eluted from A1BG7 or B1BE2 Beads with Triton X-114 in PME vs. Elution Time. (A) 0.4 mg A1BG7 or (B) 0.4 mg B1BE2 beads, preincubated with yeast soluble extract, were exposed to 300 μl 0.1% Triton X-114 in PME for increasing amounts of time. The amount of tubulin eluted was determined from quantitative western blots, stained with 345 or 206 antisera, which recognize alpha- and beta-tubulin, respectively, and is expressed as a fraction of tubulin immunoprecipitated by A1BG7 beads.
Purity and Quantity

Eluates produced from A1BG7 beads contained beta-tubulin enriched with respect to alpha-tubulin. Eluates from B1BE2 beads contained alpha-tubulin enriched with respect to beta-tubulin. We wished to determine the quantity and purity of tubulin in these preparations with respect to other proteins. We used a combination of approaches to test these parameters. We examined silver and coomassie-stained 7.5% polyacrylamide gels to assess purity and quantity, we used spectrophotometric assays to determine total protein concentration, and we examined relative tubulin signals on western blots. The results of these three approaches are described in detail below.

To examine the relative protein and tubulin content qualitatively, we compared protein signals on coomassie-stained 7.5% polyacrylamide gels from protein preparations at each step of the procedure to immunoprecipitate and elute alpha-tubulin from B1BE2 beads: the yeast soluble protein extract starting material, immunoprecipitation pellets from B1BE2 and Blank beads, eluates produced from a single treatment with 0.1% Triton X-114 in PME, or eluates produced from a treatment with 0.1% Triton X-114 in PM2G followed by elution in 0.1% Triton X-114 in PME. These eluates will henceforth be referred to as “first” or “second” eluates, respectively.

The results of this experiment are shown in Figure 4.8. Yeast soluble protein preparations, diluted 1/25 or 1/100 are shown in lanes 4 and 5, respectively. The tubulin band is impossible to discern in these fractions due to a large amount of total protein. The tubulin band and the IgG heavy chain band immediately beneath it are readily apparent, however, in immunoprecipitation pellets of B1BE2, but not Blank, beads, diluted 1/2 (lanes 6, 7).
Figure 4.8. Coomassie stain of protein preparations. Samples from each step of the procedure to produce eluates enriched in alpha- and beta-tubulin were examined on coomassie-stained 7.5% polyacrylamide gels. Samples shown are yeast soluble protein extracts, diluted 1/25 (lane 4) or 1/100 (lane 5), immunoprecipitations, diluted 1/2 (lanes 6, 7), first eluates from a single exposure to 0.1% Triton X-114 in PME (lanes 8, 9), and second eluates, produced from an exposure of 0.1% Triton X-114 in PM2G followed by an exposure to 0.1% Triton X-114 in PME (lanes 10, 11) from B1BE2 beads, (lanes 7, 9, 11) or Blank beads containing no antibody (lanes 6, 8, 10). Calf brain microtubule preparations (MTP), containing 0.5μg, 1.0μg, or 10 μg tubulin (lanes, 1, 2, 3 respectively) are also shown to compare protein quantities. The arrowhead indicates tubulin.
There were a number of other protein bands present in these preparations. At least some were constant between B1BE2 and Blank beads, suggesting non-specific interactions between yeast proteins and the bead matrix were responsible. First eluates from B1BE2 beads also contained a tubulin signal (lane 9), and a number of other, fainter protein bands. Most of these protein bands appeared to be non-specific for two reasons. First, they were present, albeit fainter, in eluates lanes of beads containing no anti-tubulin antibody (lane 9; see also Figure 4.9). Second, the second eluates from B1BE2 beads contained very few protein bands other than tubulin (lane 11), demonstrating that these eluates were highly enriched for tubulin.

To examine the protein contents of the eluate fractions from A1BG7, B1BE2, and Blank beads more carefully, eluates were examined with the more sensitive silver-stained gel analysis. These gels (Figure 4.9) were qualitatively similar to the coomassie-stained gels. First eluates from A1BG7, B1BE2, or Blank beads contained a number of protein bands, with a strong signal corresponding to tubulin by size present only in eluates from A1BG7 or B1BE2 beads. Treatment of antibody-beads with 0.1% Triton X-114 in PM2G eluted less tubulin than treatments with 0.1% Triton X-114 in PME, consistent with previously reported results from western blotting experiments (see Figure 4.2). A second exposure of the antibody beads, eluted tubulin, but very few other protein bands.

In order to confirm that the second elution did not alter the ratio of alpha- to beta-tubulin in the eluates, we examined the eluate fractions by western blotting. The results are shown in Figure 4.10. Confirming the silver-stain analysis (Figure 4.9) and results from other western blots (Figure 4.2), treatment with 0.1% Triton X-114 in PM2G eluted tubulin less efficiently than did 0.1% Triton X-114 in PME. A second elution with 0.1% Triton X-114, however, was consistent with the results from a first elution with 0.1% Triton X-114 (Figure 4.10).
Figure 4.9 Silver stain of eluates in first and second elutions from A1BG7 or B1BE2 Beads. (A) A1BG7 beads (lanes 1, 3, 5) or Blank beads (lanes 2, 4, 6), pre-incubated with yeast soluble extract, are either eluted with 0.1% Triton X-114 in PME (lanes 1 and 2), eluted with 0.1% Triton X-114 in PM2G (lanes 3 and 4), or eluted with 0.1% Triton X-114 in PM2G followed by an elution in 0.1% Triton X-114 in PME (lanes 5, 6). The three eluate fractions are loaded and run on 7.5% acrylamide gels and the gels silver stained.
Figure 4.9 (continued)  (B) B1BE2 beads (lanes 2, 4, 6) or Blank beads (lanes 1, 3, 5), pre-incubated with yeast soluble extract, are either eluted with 0.1% Triton X-114 in PME (lanes 1 and 2), eluted with 0.1% Triton X-114 in PM2G (lanes 3 and 4), or eluted with 0.1% Triton X-114 in PM2G followed by an elution in 0.1% Triton X-114 in PME (lanes 5, 6). The three eluate fractions were loaded, run on 7.5% acrylamide gels, and the gels silver-stained.
Figure 4.10. Western Blots of Double Elutions from A1BG7 and B1BE2 Beads. (A) A1BG7 beads or (B) B1BE2 beads were eluted with 0.1% Triton X-114 in PME (lanes 1,2), Triton X-114 in PM2G (lanes 3,4), or Triton X-114 in PM2G followed by 0.1% Triton X-114 in PME (lanes 5, 6). Western blots were stained with 345 or 206 antisera, which recognize alpha- or beta-tubulin respectively.
We obtained estimates of tubulin concentration by comparing protein signals in eluates from A1BG7 and B1BE2 beads on coomassie and silver-stained gels with known concentrations of tubulin from Calf brain microtubule preparations. Calf brain microtubule preparations, containing 0.5µg, 1.0 µg, or 10µg of protein, are shown in lanes 1, 2, and 3, respectively, of Figure 4.8. The tubulin in eluates from A1BG7 and B1BE2 beads was equal to ~1 µg of tubulin from Calf brain microtubule preparations. Based on the volume of the protein load, we calculated the tubulin concentration to be ~10 to 20 µg/ml in these eluates, which corresponded to ~1.8 to 3.6 x 10^{-7} M tubulin.

The second approach we used to determine the purity and concentration of tubulin in the eluates was to examine total protein concentrations using spectrophotometric analysis. Yeast soluble protein and the second eluate from A1BG7 or B1BE2 beads were analyzed for total protein content using an assay similar to the Bradford, but compatible with detergents (see materials and methods). To determine the enrichment of tubulin in the eluates with respect to the starting material, we also examined tubulin levels by western blotting analysis. The results are shown in Table 4.1.

**Table 4.1. Tubulin is Enriched in Eluates with Respect to Starting Material.**

<table>
<thead>
<tr>
<th>Protein concentration (in mg/ml)*</th>
<th>Relative tubulin concentration**</th>
<th>Fold enrichment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Extract</td>
<td></td>
<td>---</td>
</tr>
<tr>
<td>A1BG7 Eluates</td>
<td>&lt; 0.005</td>
<td>0.25</td>
</tr>
<tr>
<td>B1BE2 Eluates</td>
<td>&lt; 0.005</td>
<td>0.54</td>
</tr>
</tbody>
</table>

*Based on protein quantification assays using the BioRad DC Protein assay.
**Based on signals from quantitative western blots.
Tubulin signals from eluates were near the limit of resolution in the spectrophotometric assay, at less than 5 \( \mu \text{g/ml} \) of protein, which corresponded to \( \sim 1 \times 10^{-7} \text{ M} \) tubulin. By comparing the tubulin western signal from the eluates with the tubulin western signal from yeast whole cell extracts, we calculated a greater than 500-fold enrichment for tubulin in A1BG7 eluates and greater than 1000-fold enrichment in B1BE2 eluates compared to the starting material. Since tubulin concentrations in the starting material were estimated at \( \sim 10 \mu \text{g/ml} \) (Pillus and Solomon, unpublished), the tubulin in eluate preparations were likely greater than 90% pure.

The third method we used to determine the quantity of tubulin in eluates from A1BG7 and B1BE2 beads was western blot analysis. We compared the tubulin western signals from eluates with tubulin western signals from known quantities of purified yeast tubulin (Barnes, et al., 1992). This approach yielded values of \( \sim 40 \mu \text{g/ml} \) to \( \sim 70 \mu \text{g/ml} \), which corresponded to \( \sim 1 \times 10^{-6} \text{ M} \) tubulin in the eluates from A1BG7 and B1BE2 beads, respectively. This method also provided support for the estimate of the total tubulin concentration in yeast whole cell extracts. Previous estimates were \( \sim 10 \mu \text{g/ml} \) (L. Pillus and F. Solomon, unpublished). Using comparative western blotting analysis, we obtained values of 5 \( \mu \text{g/ml} \) to 10 \( \mu \text{g/ml} \) of tubulin in the starting material.

The results from three different approaches, comparative signals on stained gels, spectrophotometric analysis, and comparative western blotting, yielded similar but not identical values, ranging from 5 \( \mu \text{g/ml} \) to 70 \( \mu \text{g/ml} \), for tubulin concentration in eluates from A1BG7 and B1BE2 beads. At least some of the variability in these values was due to the different elution parameters used in each experiment. The eluates examined by comparative western blot were produced with a low volume of eluant, while eluates in the spectrophotometric analysis were produced from larger eluant volumes. Eluates in the silver- and coomassie-stained gels were acquired with an intermediate volume of eluant. The total amount of beads in each elution condition also varied. Consistent with this explanation, the relative western signals between eluates and the starting material were 3 to 5-fold higher in comparative western blot experiments than the values shown in Table A1, showing that there was more tubulin in these fractions.
To summarize, double eluates from A1BG7 and B1BE2 beads contained tubulin protein at concentrations from 5 μg/ml to 70μg/ml, dependent on the particular elution conditions used, which corresponded to $1 \times 10^{-7}$ M to $1 \times 10^{-6}$ M tubulin. These preparations were extremely pure with respect to other proteins. Calculating from total eluate volumes, 1 mg of A1BG7 or B1BE2 antibody produced ~20 μg of tubulin. Since elution efficiency was approximately 25%, these data were quite consistent with the values discussed above, that estimated ~80 μg of tubulin bound by 1 mg of antibody beads.
Discussion

We examined a number of experimental parameters that affected the immunoprecipitation of alpha- and beta-tubulin from *Saccharomyces cerevisiae* whole cell extracts by the anti-alpha-tubulin monoclonal antibody A1BG7 and the anti-beta-tubulin monoclonal antibody B1BE2. These parameters included the production of whole cell extracts, including the buffer composition, preparation of the antibody:bead matrix, time of binding, and the saturation state of the antibodies. Alterations at each of these steps had consequences for the immunoprecipitation of alpha- and beta-tubulin. The results obtained in the experiments testing these parameters enabled us to establish a rigid, reproducible procedure for the immunoprecipitation of alpha- and beta-tubulin with A1BG7 and B1BE2 antibodies.

We also tested a number of experimental parameters with dramatic effects on the elution of alpha- and beta-tubulin from B1BE2 or A1BG7 beads. These parameters included the eluant buffer composition, the eluant volume, and the elution incubation time. The results of these experiments provided clues about the mechanism responsible for the detergent-mediated separation of alpha- and beta-tubulin from the tubulin heterodimer. In addition, they enabled us to manipulate conditions to alter the concentration and amount of tubulin eluted, making the procedure flexible for application to functional studies of the alpha- and beta-tubulin polypeptides.

We hypothesized that the detergent-mediated separation of alpha- and beta-tubulin was due to the dissociative effects of detergent on the alpha/beta tubulin heterodimer. Several lines of evidence support this hypothesis. First, a number of reports from the literature have established that some detergents destabilize the tubulin heterodimer (Andreu, 1982; Andreu, 1986a; Andreu and Munoz, 1986b; Andreu, *et al.*, 1986c). Although direct tests of the effects of Triton X-114, Triton X-100, and NP-40 detergents on the tubulin heterodimer dissociation constant are not possible because of the spectrophotometric background produced by the detergent structures, it is likely these detergents produce similar alterations. Second, a number of laboratories have hypothesized that the tubulin
heterodimer is held together by hydrophobic forces (Prasad, et al., 1986; Sackett, et al., 1990; Sackett and Lippoldt, 1991). Non-ionic detergents could destabilize the heterodimer interactions, shifting the dissociation constant in favor of dissociation. Third, components such as GTP and glycerol, which have stabilizing effects on the tubulin heterodimer (Shearwin, et al., 1994a), prevent or decrease the efficiency of the detergent-mediated separation of alpha- and beta-tubulin.

The destabilizing effects of detergents on the tubulin heterodimer are surprising, since non-ionic detergents do not inhibit the polymerization of tubulin heterodimer into microtubules (Friden, et al., 1987). In addition, the buffer conditions are milder than buffer conditions used in standard immunoprecipitation protocols. These data suggest the alterations in heterodimer stability may be subtle. Consistent with this interpretation, Triton X-114 has only modest affects on tubulin colchicine binding (Andreu, 1982). In addition, the concentrations of tubulin produced in the detergent-mediated elution procedures are only modestly greater than the heterodimer dissociation constant concentration (Shearwin, et al., 1994a), suggesting a modest affect on the heterodimer interaction.

The separation of alpha/beta heterodimer may be enhanced by the presence of anti-alpha-tubulin and anti-beta-tubulin antibodies. Although none of the experiments described in this chapter rule out this interpretation, it seems unlikely. The epitope of the B1BE2 monoclonal antibody is located at the extreme carboxy-terminus of the yeast beta-tubulin. If this region were involved or required for heterodimer stability, changes in the primary sequence in this region would have extreme phenotypic consequences. However, deletions of this sequence in the sole beta-tubulin of S. cerevisiae had only mild phenotypic consequences (Katz and Solomon, 1988). More likely, the antibodies provide a mechanism to hold captive one of the heterodimer chains, thereby freeing the other chain to dissociate into the eluant buffer.

A number of mysteries related to the elution of tubulin from anti-tubulin beads remain. Eluates from B1BE2 beads consistently contained greater amounts of beta-tubulin, the antibody-specific tubulin chain. The unusual elution patterns for B1BE2 beads were evident in other experiments as well. B1BE2 eluates produced from exposure to 0.1% Triton X-114 in PM2G had reduced amounts of alpha-tubulin eluted relative to eluates produced in 0.1% Triton X-114/PME.
buffer. However, the amount of beta-tubulin, the antibody-specific chain, also increased (See Figure 4.2). In addition, parameters altering elution efficiency affected B1BE2 bead eluates differently than A1BG7 bead eluates. First, increasing the concentration of non-ionic detergent increased the amount of alpha-tubulin eluted from B1BE2 beads, but it also increased the amount of beta-tubulin contaminant (see Figure 4.4). Second, increased eluant volumes in elutions from B1BE2 beads increased the total amount of beta-tubulin contaminant eluted, but the concentration of beta-tubulin in the eluate remained constant (Figure 4.6). In contrast, increasing the incubation time did not increase the amount of contaminant beta-tubulin present in B1BE2 eluates (Figure 4.7). Eluates from A1BG7 beads did not exhibit these alterations in the elution of the antibody-specific alpha-tubulin chain. These results suggest a concentration-dependent, but incubation time independent, phenomena responsible for the elution of beta-tubulin from the anti-beta-tubulin B1BE2 beads. The implication of these data is that non-ionic detergents might interfere with the antibody:antigen complex, an unlikely scenario given the strong association constants typical of antibodies.

Finally, we established by a number of tests that the eluate fractions were highly enriched for alpha- or beta-tubulin, both with respect to the heterodimeric partner, and with respect to other proteins. This enabled us to perform a number of experiments testing the functional properties of alpha- and beta-tubulin monomers, as described in Chapter Three and the Appendix of this dissertation.
Chapter Four

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Chapter Five

Conclusions
Microtubules are complex polymeric structures utilized by cells in a number of diverse and complex ways. The primary subunit of the microtubule polymer is the tubulin heterodimer, composed of two similar but not identical subunits, alpha- and beta-tubulin (Raff, et al., 1987). While a number of studies have examined the structure and biochemistry of the tubulin heterodimer and microtubule polymer, the precise structure and function of the individual tubulin polypeptides chains are still poorly understood. Alpha- and beta-tubulin have proved exceedingly difficult to purify or produce in vitro in quantities that permit analysis of the biochemical and structural roles of the native chains. As a consequence, biochemical studies have been limited to exploring interactions in the heterodimer, and extrapolating to the individual chains. Crystal structures, which would provide extensive structural information about alpha- and beta-tubulin, have not been reported for these polypeptides.

Several lines of evidence demonstrate the alpha- and beta-tubulin polypeptides have divergent functional characteristics. First, over-expression of TUB2, the S. cerevisiae beta-tubulin gene, on a Galactose promoter rapidly killed cells (Burke, et al., 1989; Weinstein and Solomon, 1990). At early time points, strains over-expressing TUB2 lost all microtubule staining. At later timer points, beta-tubulin staining reappeared, in dense structures that co-localized with yeast spindle pole bodies. In contrast, much higher levels of alpha-tubulin were required to produce a lethal phenotype, and no alpha-tubulin dense structures were apparent in over-expressing strains (Weinstein and Solomon, 1990);(Margaret Magendantz, unpublished data). Since beta-tubulin over-expression was phenotypically unique from alpha-tubulin over-expression, the beta-tubulin polypeptide likely participates in unique structural interactions. Second, and consistent with the yeast over-expression data, structural analysis of the microtubule polymer placed the beta-tubulin chain at the minus end of the microtubule (Song and Mandelkow, 1995), where it likely directly interacts with microtubule nucleators. Third, gamma-tubulin, a protein with some homology to alpha- and beta-tubulin that may act as a microtubule nucleator, was identified as a suppresser of beta-tubulin mutations in A. nidulans (Oakley and Oakley, 1989). Taken together, these data reveal that beta-tubulin has a unique functional role
within the microtubule polymer, possibly interacting with nucleators or other proteins.

Study of tubulin message degradation and \textit{in vitro} translation of tubulin protein also reveal unique characteristics for the individual tubulin chains. First, experiments examining chaperonin interactions with alpha- and beta-tubulin identified several protein co-factors required for release from the chaperonin complex. The release of alpha-tubulin from the chaperonin complex required exogenous microtubules and co-factor A and B. In contrast, non-functional beta-tubulin was released from chaperonin complexes by exposure to co-factor A alone (Gao, \textit{et al.}, 1993). These data suggest a unique interaction between beta-tubulin and co-factor A. Second, microtubule depolymerizing drugs induced changes in the stability of the alpha- and beta-tubulin message of tissue culture cells (Ben-Ze'ev, \textit{et al.}, 1979; Cleveland, \textit{et al.}, 1981). Oddly, the regulation of message for alpha- and beta-tubulin appeared to operate through different mechanisms since a tetra peptide that was sufficient for beta-tubulin message regulation did not regulate alpha-tubulin message (Bachurski, \textit{et al.}, 1994). Although the precise physiological role of tubulin message autoregulation was unclear, it was surprising that alpha- and beta-tubulin were not regulated in the same way. Perhaps, autoregulation of beta-tubulin message, which requires translation initiation (Theodorakis and Cleveland, 1992), is somehow linked to the unique beta-tubulin chaperonin interactions.

Biochemical experiments provided a third line of evidence that beta-tubulin has a unique functional or structural role in the cell. First, the anti-mitotic drug colchicine was covalently cross-linked to the beta-tubulin polypeptide chain in cross-linking experiments (Wolff, \textit{et al.}, 1991). In addition, the presence of colchicine made beta-tubulin, not alpha-tubulin, more susceptible to proteolysis, possibly due to localized unfolding of the beta-tubulin polypeptide (Sackett and Varma, 1993). Other microtubule interacting drugs, such as benomyl or taxol, are less well characterized but may exhibit similar interactions. Second, in tubulin GTP binding studies, GTP was covalently cross-linked to beta-tubulin (Hesse, \textit{et al.}, 1985; Nath and Himes, 1986). Although these studies did not preclude the contribution of alpha-tubulin to binding, they did suggest the
relationship between beta-tubulin and the cross-linked material was different from that of alpha-tubulin.

The precise differences in alpha- and beta-tubulin structure, and the effects these have for microtubule function are unclear. The work described in this dissertation was intended to examine the structural relationship between alpha- and beta-tubulin, and to identify unique functional roles for each of the tubulin polypeptide chains. Using two entirely different approaches, one genetic and one biochemical, we sought to determine how the alpha- and beta-tubulin polypeptides were different from one another, and what each contributed to microtubule function.

Research from Elizabeth Raff's laboratory identified a mutation, B2t^8, in the testes-specific beta-tubulin of *D. melanogaster* with an intriguing structural phenotype. All microtubule structures of cells expressing the B2 gene were disrupted. In cross-sections of the axoneme, microtubules were S-shaped rather than 8-shaped. This phenotype suggested an alteration in the alpha-beta-tubulin heterodimer, or in the interactions between heterodimers within the microtubule polymer (Fuller, *et al.*, 1987; Rudolph, *et al.*, 1987). Lending support to this hypothesis, recent structural models of the axoneme predicted the formation of a seam when the microtubule sheet closed into a tube. Improper closure of the microtubule seam would result in S-shaped microtubules (Song and Mandelkow, 1995), precisely the phenotype observed in *D. melanogaster*.

The B2t^8 mutation was sequenced as a glutamic acid to lysine substitution at position 288 of beta-tubulin. Since the residue at position 288 of beta-tubulin is a glutamic acid in more than 40 beta-tubulins sequenced thus far, with only leishmania, chicken, and human isoforms containing an aspartic acid residue instead, it lent credence to the hypothesis that the residue was critical for microtubule structure.

We generated the same mutation in *S. cerevisiae* with the goal of analyzing a similar phenotype in yeast and performing suppressor analysis. Surprisingly, the glutamic acid to lysine substitution in *S. cerevisiae* had only a mild phenotypic consequence, a slight increased sensitivity to the anti-mitotic drug benomyl. It was unclear, given the severity of the phenotype in *D. melanogaster*, why the phenotype in *S. cerevisiae* was not more severe. It may be that the cellular
conditions within *D. melanogaster* testes are slightly different from *S. cerevisiae*, making these microtubules more sensitive to changes. These results highlight the difficulties in using *S. cerevisiae* genetics to address structural questions.

The second approach we took to examine alpha-beta-tubulin heterodimer interactions was biochemical. We developed, characterized, and optimized an *in vitro* procedure to produce protein preparations enriched for monomeric alpha- and beta-tubulin. The basic approach was simple: immunoprecipitation of tubulin heterodimer by antibodies to either tubulin chain, followed by elution of the polypeptide chain not recognized by the antibody with non-ionic detergents. The resultant protein preparations were relatively uncontaminated by other proteins, and enriched for one of tubulin polypeptide chains with respect to the other.

We used three methods, based on well-characterized tubulin polypeptide functions, to examine whether the alpha- and beta-tubulin polypeptide chains produced by the detergent-mediated separation of tubulin heterodimer retained native function. The tubulin heterodimer binds 1 mol of GTP exchangeably (Weisenberg, 1972), with beta-tubulin cross-linked to GTP in photo-cross-linking assays (Hesse, *et al*., 1985; Nath and Himes, 1986). Using the UV-cross-linking procedure, we determined whether the tubulin polypeptides produced from the detergent-mediated separation of tubulin heterodimer could bind GTP. We also examined whether the tubulin heterodimer could be reconstituted from the separated chains, as assayed by co-immunoprecipitation experiments and native gel electrophoresis analysis.

The results of the heterodimer reconstitution analysis, discussed in the Appendix of this dissertation, were ambiguous. However, the results of the GTP binding analysis were quite promising. Native tubulin levels of GTP binding activity could be reconstituted in the separated chains when the chains were recombined in equimolar concentrations. These results demonstrated that, at least by one criteria, the tubulin chains were not irreversibly denatured.

The procedure to isolate monomeric alpha- and beta-tubulin has a number of potential applications. Colchicine (Sackett and Varma, 1993; Wolff, *et al*., 1991), co-factor A (Gao, *et al*., 1993), and other proteins have been identified which may interact preferentially with one of the tubulin heterodimer chains. Using pools of protein that are enriched for alpha- or beta-tubulin, the precise interactions
between these components and the microtubule polypeptides can be established.

Other components, such as the anti-mitotic drug benomyl, suggest a direct interaction between alpha- or beta-tubulin. Using the detergent-mediated separation of alpha- and beta-tubulin, direct interactions between these polypeptides could be established and characterized.

The protein pools containing alpha- and beta-tubulin are relatively uncontaminated by other proteins. This result makes it possible to use this assay to identify proteins that interact preferentially with alpha- or beta-tubulin. Since the protocol was developed in yeast, genetic analysis could be used to complement the biochemical analysis.

Finally, production of highly enriched preparations of alpha- and beta-tubulin using detergent-mediated separation of alpha- and beta-tubulin may be the first step to purifying, and eventually crystallizing, these polypeptides. The structural information obtained from such studies would produce tremendous insight into the roles of alpha- and beta-tubulin within the heterodimer, and the microtubule polymer.
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Appendix
Introduction

Chapter Three of this dissertation described a protocol utilizing the detergent-mediated separation of the alpha- and beta-tubulin heterodimer to produce relatively pure preparations of alpha- and beta-tubulin monomeric polypeptide. The detergent-mediated procedure used gentle conditions, low concentrations of non-ionic detergent in microtubule stabilizing buffer, that were compatible with microtubule polymerization (Friden, et al., 1987). However, we were concerned that the conditions of the procedure might have rendered the monomeric alpha- and beta-tubulin polypeptides non-functional. We decided to determine if the alpha- and beta-tubulin polypeptides were irreversibly denatured.

There are several properties characteristic of native alpha- and beta-tubulin chains that can be tested, including the formation of the tubulin heterodimer and GTP binding. The presence of the tubulin heterodimer was established by a number of indirect experiments, including cross-linking experiments, co-immunoprecipitations, genetic mutant analysis, and native gel electrophoresis (Lee, et al., 1973; Luduena, et al., 1977; Stearns and Botstein, 1988). We chose two of these assays, co-immunoprecipitation and native gel electrophoresis, as methods to determine whether alpha- and beta-tubulin produced by the detergent-mediated separation of tubulin heterodimer could form heterodimers. The results of these experiments are described in this appendix.

A second well-established biochemical function of native tubulin heterodimer is GTP binding. Using cross-linking experiments, a number of laboratories have established that the beta-tubulin subunit is a critical component of GTP binding (Geahlen and Haley, 1977; Hesse, et al., 1985; Hesse, et al., 1987; Mitchison, 1993) However, the precise contribution of alpha-tubulin, if any, to GTP binding has not been examined in any detail. We chose to examine the GTP binding activities of alpha- and beta-tubulin obtained from the detergent-mediated separation of alpha- and beta-tubulin. Many of the results of these experiments are described Chapter Three of this dissertation. However, several important experiments and controls which could not be included in that chapter are included here.
Materials and Methods

Strains, Media, and Culture Conditions

The *S. Cerevisiae* strains used in this study were CKY93, (MATα; leu2-3,112, pep4::URA3; ura3-52), and FSY127 (ura3-52, lys2-801, leu2-3,112, tub2-590). CKY93 contains a disruption of the PEP4 locus, which reduces endogenous protease activity. FSY127 has a twelve amino-acid carboxy-terminal truncation in TUB2, the only beta-tubulin gene in *S. cerevisiae* (Katz and Solomon, 1988). The strains were grown in synthetic complete media minus amino-acids specific for the strain auxotrophic marker (Katz, *et al.*, 1990; Sherman, *et al.*, 1986). Hybridomas were cultured as described in Chapter Four of this dissertation.

Monoclonal Antibody Generation

The two peptides used to generate monoclonal antibodies were NH2-CADSYAAEEE-COOH (TUB1C peptide; Biopolymers Laboratory, Center for Cancer Research, M. I. T.) and NH2-CQNQDEPITENFE-COOH (TUB2C peptide; Clontech, Palo Alto, California). Antibody generation is described fully in Chapter Four of this dissertation. A1 BG7 secreted an IgG3 with a k light chain. B1 BE2 secreted an IgG2a with a k light chain. Ascites fluid was produced from these hybridomas by Charles River Laboratories (Wilmington, MA). IgG was purified from ascites fluid using a ProteinA IgG purification kit (Pierce, Rockford, IL), and covalently coupled overnight to Affigel-10 beads (BioRad Laboratories, Richmond, CA) in 50 mM HEPES pH 6.9. 8-16 mg of protein were coupled per ml of beads. For controls, we incubated Affi-gel10 beads in buffer alone. After, incubation with antibodies or buffer from several hours to overnight, the active sites on the beads were blocked by a one hour incubation in 1M ethanolamine (MCB, Cincinnati, OH) in 50 mM Hepes buffer, pH 6.9. Antibodies coupled to beads, (henceforth referred to as A1BG7 beads, B1BE2 beads, or Blank beads), were used immediately for immunoprecipitations.

Preparation of yeast total soluble protein extracts

*S. Cerevisiae* strains were grown to late log phase, harvested by centrifugation, and washed once in cold PME buffer (0.1M Pipes pH 6.9, 1 mM MgSO4, 2 mM EGTA ). The cell pellet was resuspended in two times the pellet volume cold PME buffer with protease inhibitors (1 μg/ml each of leupeptin, phenylmethylsulfonyl fluoride (PMSF),
aprotinin, tosyl phenylalanine chloromethyl ketone (TPCK), and pepstatin (all obtained from Sigma Chemical)). The cell suspension was twice passed through a cold French Pressure apparatus (SLM Aminco, Urbana, IL), using a 20,000 PSI pressure cell with a 3/8" diameter piston at an internal pressure of 20,000 PSI. The resultant cell lysate was cleared by centrifugation at 27,000g for 15 minutes at 4°C. The pellet was discarded and the supernatant was diluted two-fold with cold PM3G (0.1M Pipes pH 6.9, 1 mM MgSO4, 2 mM EGTA, 3M Glycerol) with protease inhibitors. The soluble protein extracts were used immediately for immunoprecipitations.

**Immunoprecipitation of tubulin and isolation of tubulin monomers.**

Yeast soluble protein extracts were added in sufficient volume to saturate the anti-alpha tubulin or anti-beta-tubulin antibody coupled to beads and incubated on a tube rotator at 4°C for four hours. The beads containing immunoprecipitated tubulin were washed five times with a minimum of 10 bead volumes cold PM2G buffer with protease inhibitors and washed five times with a minimum 10 bead volumes cold PME with protease inhibitors.

Tubulin immunoprecipitated by antibody beads was incubated with 0.1% Triton X-114 (Octylphenoxypolyethoxyethanol, Sigma Chemical Co, St. Louis, MO) in PME with protease inhibitors on a tube rotator at 4°C for 90 minutes. The elution volume ranged from 0.15 ml to 1 ml per 1 mg of antibody beads. Following incubation, the supernatant containing tubulin was carefully removed. It was either diluted with 1/2 volume GSD, and boiled 5 minutes, or used immediately for additional experiments. The pellet was resuspended in PME, and boiled with 1/2 volume GSD for 5 minutes.

**SDS and Native Gel Western Blotting**

To quantitate tubulin, protein samples in GSD were loaded in duplicate or triplicate and run on 7.5% SDS-Polyacrylamide Mini-Protean gels, and transferred to 0.2 µm nitrocellulose according to the MiniProtean II manufacturers instructions (BioRad). Polyclonal anti-alpha-tubulin antibody, 345-4 (Schatz, *et al.*, 1988), and polyclonal anti-beta-tubulin antibody, 206-1 (Bond, *et al.*, 1986) were used in western blots, as previously described (Fridovich-Keil, *et al.*, 1987). Blots were exposed to a phosphorimager screen overnight, scanned, and the bands were quantitated using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The data was transferred to the spreadsheet program Excel (Microsoft, Redmond, WA). In order to
compare data from 345-4 and 206-1 western blots, all values for eluate and pellet fractions were normalized to control for loading and volume differences and expressed relative to the tubulin values in yeast whole cell extracts or in purified yeast tubulin preparations (Barnes, et al., 1992).

The procedure for native gels was essentially the same as SDS-gels. Briefly, freshly prepared protein samples were diluted in loading buffer (tris-glycine running buffer (without SDS) and with 10 μM GTP, 1 mM MgSO₄, protease inhibitors, 33% glycerol, and standard dyes) and run on 7.5% polyacrylamide gels made according to standard protocols (BioRad), except they contained no SDS and 10 μM GTP. Tris-glycine running buffer, without SDS, also contained 10 μM GTP. All buffers were chilled to 4°C and gel electrophoresis was performed at 4°C to reduce potential protein denaturation. The gel contents were immediately transferred to nitrocellulose (BioRad) and subject to western blotting analysis.

**GTP cross-linking assays**

GTP binding by photolinking was adapted from conditions used in other systems (Hesse, et al., 1985; Nath and Himes, 1986; Penefsky, 1977; Yue and Schimmel, 1977). 6 μl of extract from A1BG7 beads and 6 μl of extract from B1BE2 beads were either added to each other, or to 6 μl of 0.1% Triton X-114 in PME with protease inhibitors. As a control, purified yeast tubulin (Barnes, et al., 1992) was quickly thawed and diluted in 0.1% Triton X-114 in PME with protease inhibitors to concentrations of alpha- and beta-tubulin similar to those of the eluate fractions. A concentration range of purified yeast tubulin was tested. The samples were brought to 15 μl with 1X MES buffer (40 mM MES, pH 6.4, 0.4 mM EGTA, and 0.2 mM MgCl₂) and incubated for 30 minutes at 4°C on a tube rotator. The protein was transferred to a 96-well plate flat bottom plate (Dynatech Laboratories). 5 μl of Hot GTP mix (2X MES, 0.16 mM ATP, 3 × 10⁻⁷ M ³²P-α-GTP, 4 μM GTP) were added to each well and the samples incubated on ice for 30 minutes. The GTP was cross-linked to the proteins in a Stratalinker (Stratagene) at 254 nm wavelength at a distance of 3 cm and a total energy level of 2 x 10⁶ mJoules. The proteins were mixed 1:1 with loading buffer, run immediately on one-dimensional gels, and transferred to nitrocellulose, as described above. Protein was visualized with 345-4 and 206-1 antisera, and secondary antisera Alkaline Phosphatase conjugated goat anti-rabbit (AP-GAR). ³²P-α-GTP was visualized by exposure to Kodak X-Omat film at -70°C with a screen. To determine the amount of
cross-linked $^{32}$P-$$\alpha$$-GTP present in eluate samples, blots were exposed to a phosphorimager screen, scanned, and bands were quantitated using ImageQuant software (Molecular Dynamics) and manipulated on the spreadsheet program Excel (Microsoft).

**Co-immunoprecipitations**

A1BG7 and B1BE2 monoclonal antibodies were purified, coupled to Affi-gel 10 beads, and blocked with 1M ethanolamine, as described above. In some experiments, yeast soluble protein extracts in PM2G from FSY127 strains, which contain a deletion of the carboxy-terminal peptide of TUB2 used to generate B1BE2 monoclonal antibodies, were incubated with B1BE2 beads for 4 hours at 40$^\circ$C to block non-specific interactions. After the incubation, the B1BE2 beads were extensively washed with PME. No alpha- or beta-tubulin protein was immunoprecipitated by the B1BE2 beads under these circumstances, as assayed by western blotting.

Eluates from A1BG7 and B1BE2 beads containing predominantly beta- or alpha-tubulin, respectively, were produced as described above. The eluates were mixed 1 to 1 with buffer or with each other and 100 mM GTP and, in some cases, 8M glycerol, were added in sufficient quantities to produce 1 mM GTP and 2 M glycerol buffer. These eluates were incubated with A1BG7 beads, B1BE2 beads, or B1BE2 beads, blocked with yeast FSY127 whole cell extracts, for 4 hours if the buffer contained glycerol or 1 hour if the buffer did not. The supernatants were carefully removed and diluted 1/2 with GSD and boiled 5 minutes. The beads were washed once in PME and the wash diluted 1/2 with GSD and boiled 5 minutes. The pellets were resuspended and diluted 1/2 with GSD and boiled 5 minutes. The protein preparations were analyzed by SDS gel electrophoresis.
Appendix

Results

Native gel electrophoresis

The mobility of tubulin on native polyacrylamide gels has been examined in a number of circumstances. Typically, thrice-cycled calf brain microtubule preparations loaded onto native gels exhibited a characteristic "ladder" of tubulin, with two bands migrating distances characteristic of 55 kDa proteins, and additional bands at 110 kDa, and increasing in 110 kDa increments (Correia and Jr., 1985; Kravit, et al., 1984; Lee, et al., 1973).

We examined the mobility of tubulin in eluates from A1BG7 and B1BE2 beads on 7.5% native polyacrylamide gels. Briefly, yeast soluble protein extracts were incubated with anti-alpha-tubulin antibody A1BG7 or anti-beta-tubulin antibody B1BE2 for 4 hours. The immunoprecipitated material was washed and the tubulin eluted with 0.1% Triton X-114 in PME. The eluate fractions were mixed together or with buffer, run on 7.5% polyacrylamide native and SDS gels, and transferred to nitrocellulose. Tubulin mobility was examined by western blots stained with anti-alpha-tubulin antisera 345 or anti-beta-tubulin antisera 206.

The results of this experiment are shown in Figure A.1. SDS gel western blots (Figure A.1 B; lanes 5 - 8) showed that the tubulin proteins were well-behaved in the detergent-mediated separation protocol. Eluates from B1BE2 beads contained a significant alpha-tubulin signal, with little beta-tubulin present. Eluates from A1BG7 beads contained a significant beta-tubulin signal with little alpha-tubulin present. Mixed eluates contained significant alpha- and beta-tubulin, consistent with the sum of the tubulin from A1BG7 and B1BE2 eluates. Blank beads contained no tubulin signal.

On native gels (Figure A.1 A; lanes 5 - 8), the majority of alpha- and beta-tubulin protein in the A1BG7 or B1BE2 eluate fractions ran as a 55 kDa protein. Some higher bands, including one at 110 kDa, were detectable. Oddly, these bands were present in westerns stained with anti-alpha-tubulin antibody 345, even when no anti-beta-tubulin 206 staining was apparent, suggesting the presence of homo-dimers. In mixed eluates, where both alpha- and beta-tubulin were present in equimolar quantities, alpha- and beta-tubulin ran as 55 kDa protein, identical to the A1BG7 and B1BE2 eluate fractions. Some alpha- and beta-tubulin co-localized at 110 kDa, but it was impossible to distinguish these interactions from alpha-alpha or beta-beta interactions.
Figure A1. Native and SDS Gels of eluates from A1B7, B1BE2, Blank, and Mixed eluates. (A) Native gels or (B) SDS-gels containing eluates from A1BG7 beads (lanes 1, 5), eluates from B1BE2 beads (lanes 2, 6), mixed eluates (lanes 3, 7), or eluates from blank beads (lanes 4, 8). Eluates contained 0.1% Triton X-114 in PME (lanes 5 to 8) or the detergent was phase-shifted (lanes 1 to 4). Western blots were stained with polyclonal antisera 345 or 206, which recognized alpha- or beta-tubulin, respectively.
These data suggested that little or no heterodimer formed when eluates from A1BG7 and B1BE2 beads were mixed together. There were a number of possible explanations for this result. First, the conditions of the elution, low concentrations of non-ionic detergent in PME buffer, although extremely gentle, rendered the polypeptides incapable of forming heterodimers because they were denatured. Second, despite the inclusion of GTP/Mg++ in the gel buffers, components that stabilized the tubulin heterodimer (Shearwin, et al., 1994a), the association between alpha- and beta-tubulin was unstable under the conditions of the native gel electrophoresis. This was a particular concern because the concentrations of protein were near dissociation constant concentrations. Third, the presence of non-ionic detergents in the buffer may have destabilized the heterodimer (Andreu, 1982; Andreu, 1986a; Andreu, et al., 1989; Andreu and Munoz, 1986b; Andreu, et al., 1986c), preventing reformation of heterodimer.

Since the third hypothesis was testable, we examined the migration of tubulin in eluates from A1BG7 or B1BE2 beads after removal of the Triton X-114 detergent on native gels. Triton X-114 detergent forms a “cloud point” or, essentially, most precipitates out of solution when buffer temperatures are raised (Bordier, 1980). The particular temperatures required were shown to be dependent on detergent concentration, buffer, and salt concentrations (Doren and Goldfarb, 1970; Goldfarb and Sepulveda, 1969). We raised the buffer temperatures from 4°C to 30°C for 5 minutes when the detergent cloud point became visible. The detergent was quickly pelleted, and the supernatant, with most of the detergent removed, examined by native and SDS gel electrophoresis as previously described. The results are shown in Figure A.1.

Again, A1BG7 and B1BE2 eluates were well behaved in the detergent-mediated separation protocol, as monitored on SDS gels (lanes 1-4). Alpha- and beta-tubulin were eluted from B1BE2 or A1BG7 beads, respectively, with little cross-contamination of the tubulin chains. However, reduced detergent levels clearly altered the tubulin mobility patterns on N*ative gels (lanes 1-4). Removal of most of the Triton X-114 detergent produced tubulin species with slower mobilities in the A1BG7 and B1BE2 eluate fractions compared to eluates containing detergent. Small amounts of alpha- and beta-tubulin protein were present at 55 kDa and 110 kDa, but the majority of the protein was larger. Again, mixed eluates looked quite similar to eluates from A1BG7 or B1BE2 beads, although they exhibited a slightly higher characteristic mobility. It
was unclear whether this was due to specific alpha-beta-tubulin interactions or higher concentrations of protein contributing to additional non-specific aggregation.

The results of native gel electrophoresis experiments were a concern because they suggested it was not possible to examine alpha- and beta-tubulin produced by the detergent-mediated separation of tubulin heterodimer by standard biophysical assays. TritonX-114 detergent interferes spectrophotometrically with fluorescence or other assays because of its ring structure. The reduction of detergent in eluates resulted in extensive aggregation, at least by native gel electrophoresis assays. Aggregation also interferes with biophysical tests for tubulin polypeptide nativity. As a consequence we were unable to examine these structures using standard biophysical assays.

**Co-immunoprecipitation experiments**

Co-immunoprecipitation experiments were the second method we chose to examine the interactions between alpha- and beta-tubulin produced from the detergent-mediated separation of tubulin heterodimer. Briefly, eluates from A1BG7 or B1BE2 beads, either with or without most of the Triton X-114 detergent, were incubated mixed together or separately with A1BG7 or B1BE2 antibodies, as shown in Figure A.2. The immunoprecipitation pellets and supernatants were examined quantitatively by western blotting analysis.

The results in each case were qualitatively similar to one another, and to the native gel electrophoresis experiments. In reduced detergent, beta-tubulin in eluates from A1BG7 beads was completely immunoprecipitated by anti-alpha-tubulin A1BG7 antibodies despite insufficient quantities of alpha-tubulin to form heterodimers. In reduced detergent, all of the alpha-tubulin in eluates from B1BE2 beads was immunoprecipitated by anti-beta-tubulin antibody B1BE2 antibodies despite insufficient quantities of beta-tubulin in these eluates to form heterodimers. These results demonstrated that the free alpha- and beta-tubulin polypeptide chains interacted non-specifically with antibodies or the bead matrix with reduced Triton X-114 detergent.
Figure A.2. The basic experimental protocol for the co-immunoprecipitation of alpha- and beta-tubulin produced by the detergent-mediated separation of alpha- and beta-tubulin. Briefly, eluates from A1BG7 and B1BE2 beads, containing excess beta- or alpha-tubulin relative to their heterodimeric partners, were incubated with A1BG7 or B1BE2 beads. The resultant immunoprecipitation pellets and supernatants were examined by western blotting analysis.
We attempted to block the non-specific interaction between the antibody/beads and the tubulin polypeptides using yeast whole cell extracts containing an altered tubulin chain not recognized by the B1BE2 antibody. Briefly, the B1BE2 antibody recognized a 12 amino-acid carboxyl-terminal peptide of *S. cerevisiae* Tub2p. The *S. cerevisiae* strain FSY127 contains a deletion in beta-tubulin of the 12 amino-acid carboxy-terminal B1BE2 epitope. Under most growth conditions FSY127 grows like wildtype strains (Katz and Solomon, 1988). We made yeast soluble protein preparations from FSY127, and pre-incubated the B1BE2 beads with these extracts. The tremendous concentration of non-specific yeast protein should have blocked any bead/antibody non-specific sites.

We added A1BG7, B1BE2, or mixed eluates from which most of the Triton X-114 detergent had been removed to the pre-treated B1BE2 beads. Alpha-tubulin from B1BE2 eluates was immunoprecipitated with B1BE2 antibodies, despite insufficient quantities of beta-tubulin to form heterodimers in these preparations. Since the B1BE2 eluate control lanes contained so much alpha-tubulin, it was impossible to determine whether an enrichment for alpha-tubulin binding occurred in the mixed eluates.

Finally, we tested the Immunoprecipitation of alpha- and beta-tubulin from A1BG7, B1BE2, or mixed eluates in the presence of Triton X-114 detergent. In immunoprecipitations with blocked B1BE2 beads, more alpha-tubulin immunoprecipitated from mixed eluates than from B1BE2 controls. However, the amount of alpha-tubulin immunoprecipitated from B1BE2 eluates was high, higher than expected from estimates of the beta-tubulin contamination in these fractions, and likely due to non-specific interactions with the antibodies. As a consequence, the difference between the alpha-tubulin immunoprecipitated from mixed vs. B1BE2 eluates was not significant.

The results from co-immunoprecipitation experiments were qualitatively similar to the results from the native gel electrophoresis analysis. Free alpha- and beta-tubulin chains were susceptible to non-specific interactions with each other, and with other proteins.
GTP Binding

The third test for function of the alpha- and beta-tubulin produced from A1BG7 and B1BE2 eluates relied on a biochemical rather than physical interaction. GTP binding to tubulin has been extensively characterized. A number of experiments suggest beta-tubulin plays an essential role in tubulin GTP binding, including cross-linking experiments (Lee, et al., 1973; Luduena, et al., 1977; Stearns and Botstein, 1988) and S. cerevisiae genetic mutant analysis (Davis, et al., 1994). However, these experiments did not determine whether beta-tubulin was sufficient for GTP binding. We chose to examine whether beta-tubulin produced from the detergent-mediated separation of tubulin heterodimer could bind GTP and whether the interaction required alpha-tubulin.

We tested GTP binding in the alpha- and beta-tubulin produced by the detergent-mediated separation of alpha- and beta-tubulin heterodimer using a GTP photo cross-linking assay. The basic protocol for these experiments is diagrammed in Figure A.3. Briefly, eluates from A1BG7 or B1BE2 beads, or purified yeast protein, were mixed with buffer or together and incubated for 30 minutes on ice. $^{32}$P-α-GTP was incubated with the three eluate fractions for an additional 30 minutes on ice. The eluates were transferred to 96-well plates, UV-cross-linked, run on 7.5% polyacrylamide gels, and transferred to nitrocellulose. The blots were probed with 206 or 345, which recognized beta- or alpha-tubulin respectively, and an Alkaline phosphatase-conjugated secondary antibody. To visualize $^{32}$P-α-GTP activity, the blots were exposed to a phosphorimager screen.

The resultant data from these experiments were examined extensively in Chapter Three of this dissertation. However, several important controls could not be contained in Chapter Three and they are included here.

First, only a single GTP signal was present in lanes containing cross-linked tubulin, and the cross-linked GTP signal co-migrated with tubulin. A1BG7, B1BE2, or mixed eluates were incubated with $^{32}$P-α-GTP, photo-cross-linked and run on one-dimensional SDS gels. Westerns were performed with alkaline-phosphatase-
Figure A.3. GTP binding to tubulin produced by the detergent-mediated separation of alpha- and beta-tubulin heterodimer. Eluates from A1BG7 or B1BE2 beads were produced as described previously. The eluates were incubated with buffer, or mixed together, for 30 minutes on ice. $^{32}$P-GTP was incubated together with the eluates for an additional 30 minutes. The eluates were cross-linked, run on polyacrylamide gels, transferred, and examined by western blot and phosphorimager analysis.
Figure A.4. $^{32}$P-GTP Co-migrated with Tubulin on One-dimensional Gels after Photo-Crosslinking. Mixed eluates (lane 1), B1BE2 eluates (lane 2), or A1BG7 eluates (lane 3) were incubated with $^{32}$P-GTP. The $^{32}$P-GTP was covalently cross-linked to the samples and the proteins separated by one-dimensional 7.5% SDS-polyacrylamide gel electrophoresis. Beta-tubulin migration was established by staining blots with anti-beta-tubulin antibody 206. Alpha- and beta-tubulin co-migrated under these conditions. The migration of $^{32}$P-GTP was determined by exposure to film.
Appendix

conjugated secondary antibody to produce a color reaction. $^{32}$P-α-GTP signals were obtained by exposing the western blots to film. Figure A.4 shows the results of a $^{32}$P-α-GTP cross-linking experiment. The $^{32}$P-GTP signal co-migrated with tubulin and no other $^{32}$P-GTP signal was present in these preparations.

Second, we wanted to establish that the GTP binding in purified yeast tubulin was linear with respect to protein over the range of tubulin protein concentrations tested. A ten-fold concentration range of purified yeast protein preparations (Barnes, et al., 1992) were incubated with $^{32}$P-GTP and UV cross-linked according to standard protocols. $^{32}$P-GTP signal was quantified by exposing western blots containing cross-linked protein to a phosphorimager screen. Alpha- and beta-tubulin protein values from the purified yeast protein preparations were determined separately, by western blot analysis. The results of these experiments are shown in Figure A.5.

The GTP binding signal for yeast soluble protein extracts was linear over most of the ten-fold range, as was tubulin protein signal. At high concentrations, both the protein and corresponding GTP signals leveled off. This result likely reflects saturation of the nitrocellulose, or incomplete transfer of protein from the gels. Experiments examining GTP binding in this dissertation remained in the linear range of protein-GTP signals.
Figure A. 5. GTP Binding to Purified Yeast Tubulin Preparations. A ten-fold concentration range of purified yeast tubulin was examined for GTP binding using a photo cross-linking assay. Total $^{32}$P-GTP signal was determined from phosphorimager analysis. The amount of alpha- and beta-tubulin protein present in these preparations was determined from quantitative western blotting, visualized with the anti-alpha-tubulin antibody 345 or the anti-beta-tubulin antibody 206. Only the values for alpha-tubulin are shown, although the results for beta-tubulin were consistent.
Discussion

The precise functional differences between the alpha and beta-tubulin subunits of the tubulin heterodimer are not known, in part because it has not been possible to produce pure functional preparations of these polypeptides in vitro. We have developed a new in vitro procedure, described extensively in chapter four of this dissertation, to produce protein preparations enriched for alpha- or beta-tubulin monomers. To ensure that the tubulin polypeptides produced using this technique were functional, we examined the capacity of the tubulin chains to form heterodimers, by co-immunoprecipitation experiments and native gel electrophoresis migration, and to bind GTP, using a GTP photo-cross-linking assay.

The first two approaches, native gel electrophoresis and co-immunoprecipitation experiments, yielded qualitatively similar results. The isolated alpha- and beta-tubulin chains were prone to non-specific interactions with the antibody chains and the bead matrix in co-immunoprecipitation experiments. In native gel electrophoresis, the individual chains formed homodimers and homo-aggregates, likely due to non-specific interactions with one another. The presence of the heterodimeric partner tubulin chain in these preparations did not significantly alter the native gel electrophoresis patterns. Interactions between native alpha- and beta-tubulin chains might produce similar patterns. Certainly similar native gel electrophoresis experiments using purified calf brain microtubules have also yielded complex patterns for the tubulin polypeptides (Lee, et al., 1973).

These results have important implications for experiments using the detergent-mediated separation of tubulin heterodimers to identify proteins that interact with one of the heterodimer polypeptide chains. It is critical that careful control experiments be performed to eliminate the possibility of identifying non-specific interactions. In addition, the aggregation and non-specific interactions between the individual tubulin chains make it difficult to perform biophysical analysis of the polypeptides prepared in this manner. Future research may yield improvements on the technique to separate the tubulin polypeptides chains, allowing these experiments to be performed.
Finally, we examined the GTP binding to the individual polypeptide chains. This research was described extensively in Chapter Three. Native GTP binding activity could be reconstituted from the separated chains by recombining them in 1 to 1 molar ratios demonstrating that the tubulin chains retained sufficient native secondary structure to reconstitute the tubulin GTP binding pocket. These results suggested, at least by this criteria, that the chains were not irreversibly denatured.
Appendix

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