Site-Directed Mutagenesis of Putative GTP-Binding Sites of Yeast β-Tubulin: Evidence That α-, β-, and γ-Tubulins Are Atypical GTPases†

Carleton R. Sage,‡,§ Cynthia A. Dougherty,‡ Ashley S. Davis,‡,∥ Roy G. Burns,⊥ Leslie Wilson,‡ and Kevin W. Farrell*,‡

Department of Biological Sciences, University of California, Santa Barbara, California 93106, and Biophysics Section, The Blackett Laboratory, Imperial College of Science, Technology and Medicine, London SW7 2BZ, U.K.

Received February 9, 1995; Revised Manuscript Received March 30, 1995⊗

ABSTRACT: The exchangeable GTP-binding site on β-tubulin has been extensively studied, but the primary sequence elements which form the binding site on β-tubulin remain unknown. We have used site-directed mutagenesis of the single β-tubulin gene of *Saccharomyces cerevisiae* to test a model for the GTP-binding site on β-tubulin, which was based on sequence comparisons with members of the GTPase superfamily [Sternlicht, H., Yaffe, M. B., & Farr, G. W. (1987) *FEBS Lett. 214,* 226−235]. We analyzed the effects of D295N, N298K, and N298Q mutations in a proposed base-binding motif, ^{295}DAKN^{298}, on tubulin−GTP binding and on nucleotide-binding specificity. We also examined the effects of a D203S mutation in a putative phosphate-binding region, ^{203}DNEA^{206}, on nucleotide binding affinity, on the assembly-dependent tubulin GTPase activity *in vitro*, and on the dynamic properties of individual "mutant" microtubules *in vitro*. The effects of the mutations on cell phenotype and on microtubule polymerization in cells were also measured. The results do not support the proposal that the ^{203}DNEA^{206} and ^{295}NKAD^{298} motifs are cognate to motifs found in GTPase superfamily members. Instead, the data argue that the primary sequence elements of β-tubulins that interact with bound nucleotide, and presumably also those of the α- and γ-tubulin family members, are different from those of "typical" GTPase superfamily members, such as p21^{ras}. The GTPase superfamily should thus be broadened to include not just the typical GTPases that show strong conservation of primary sequence consensus motifs (GxxxxGK, T, DxxG, DxKN) but also "atypical" GTPases, exemplified by the tubulins and other recently identified GTPases, that do not show the consensus motifs of typical GTPases and which also show no obvious primary sequence relationships between themselves. The tubulins and other atypical GTPases thus appear to represent convergent solutions to the GTP-binding and hydrolysis problem.

The tubulin family of proteins, the α-, β-, and γ-tubulins, are members of a large family of proteins which bind and hydrolyze guanosine triphosphate (GTP). The α- and β-tubulins normally associate to form a heterodimer which binds 2 mol of GTP (Jacobs, 1975; Luduena et al., 1977). Cross-linking (Geahlen & Haley; 1977; Maccioni & Seeds, 1983; Nath et al., 1985; Steiner, 1984) and immunological (Hesse et al., 1985) studies have shown that GTP binding to one site (the E-site) is readily exchangeable and is located on β-tubulin. By contrast, GTP is bound nonexchangeably at the second site (the N-site) and is presumed to be on the α-subunit. Furthermore, only β-tubulin is a *bona fide* GTPase and hydrolyzes GTP during microtubule formation [reviewed in Dustin (1984)]; GTP hydrolysis by α-tubulin has not been observed, and the nucleotide-binding and hydrolysis properties of γ-tubulin are not known. Somewhat unusual, however, is the observation that none of the tubulins show the characteristic arrangement of primary sequence motifs common to virtually all other members of the GTPase superfamily (Bourne et al., 1991; Burns et al., 1993) and which crystallographic studies have confirmed are involved in nucleotide binding (Jurnak, 1985; Pai et al., 1990; Noel et al., 1993).

Particular interest has focused on the β-tubulin GTPase because of its proposed role in microtubule dynamic properties and in the cellular functions of microtubules (Mitchison & Kirschner, 1984; Kirschner & Mitchison, 1986). Since it has not been possible to crystallize the tubulins, attempts to identify β-tubulin sequences involved in GTP binding have been limited primarily to nucleotide cross-linking studies and to sequence comparisons with GTPase superfamily members. Unfortunately, these studies from several laboratories have not provided a coherent picture but instead have identified many different regions (Figure 1). Amino acids β3−19 have been identified by two groups using UV cross-linking of unmodified GTP (Shivanna et al., 1993) or 8-azido-GTP (Jayaram & Haley, 1994) to the tubulin dimer. It has also been proposed that part of this region is homologous to the consensus GXXXXGK phosphate-binding motif found in other members of the GTPase superfamily (Shivanna et al., 1993). The β-tubulin sequences in this region, however, are not conserved in all β-tubulins (Burns, 1991; Little & Seehaus, 1988), as might be expected of a region involved in such a highly conserved function. Another study, which

† This work was supported by NIH Grants GM41751 (K.W.F.) and NS13560 (L.W.).

* To whom correspondence should be addressed [telephone, (805) 893-3851; FAX, (805) 893-4724].

‡ University of California, Santa Barbara.

§ Present address: Department of Biochemistry and Biophysics, Box 0448, University of California, San Francisco, CA 94143-0448.

∥ Present address: Cytoskeleton, 1899 Gaylord St., Denver, CO 80206.

⊥ Imperial College of Science, Technology and Medicine.

⊗ Abstract published in *Advance ACS Abstracts,* May 15, 1995.

0006-2960/95/0434-7409$09.00/0

Table 1: Yeast Strains Used in This Study

strain	β-tubulin genotype	selection marker genotype	reference
FY41	*TUB2*	*leu2d1 trp1d63 ura3-52 his4-917*	F. Winston, personal communication
ADY101	*tub2-590/tub2-590*	*HIS4/his4 leu2/leu2 LYS4/lys4 trp1/trp1 ura3/ura3*	Sage et al., 1995
ADY103	*TUB2/TUB2*	*HIS4/his4 leu2/leu2 LYS4/lys4 trp1/trp1 ura3/ura3*	Sage et al., 1995
CSY3	*tub2-590/tub2-590::LEU2*	*HIS4/his4 leu2/leu2/LEU2 LYS4/lys4 trp1/trp1 ura3/ura3*	Sage et al., 1995
N298K	*tub2-N298K*	*leu2d1 trp1d63 ura3-52 his4-917 URA3*	this study
N298Q	*tub2-N298Q*	*leu2d1 trp1d63 ura3-52 his4-917 URA3*	this study
CSY3 + pCS3-D295N	*tub2-D295N/tub2-590*	*HIS4/his4 leu2/leu2/LEU2 LYS4/lys4 trp1/trp1 ura3/ura3/URA3*	this study
ADY101-D203S	*tub2-D203S/tub2-590*	*HIS4/his4 leu2/leu2 LYS4/lys4 trp1/trp1 ura3/ura3/URA3*	this study

also used direct cross-linking of GTP to the tubulin dimer, identified the region consisting of amino acids β63−77 (Linse & Mandelkow, 1988); this region was also identified using a photoaffinity analogue of GTP (Chavan et al., 1990). It has been proposed that this region is similar to a nucleotide-binding domain of EF-Tu (Linse & Mandelkow, 1988), although the homology is weak. Yet a third region, consisting of amino acids β155−174, was also identified in a GTP-cross-linking study (Hesse et al., 1987). This region shows no direct homology with other nucleotide-binding proteins but is located just C-terminal to a region of β-tubulin that is very similar to other nucleotide-binding proteins (Baker et al., 1992).

A second approach to identifying β-tubulin GTP-binding domains has involved comparisons of β-tubulin primary sequences with those of other nucleotide-binding proteins. By comparison with EF-Tu, Linse and Mandelkow (1988) identified the region ^{60}KYVPRAILVD^{69} of β-tubulin as being involved with guanine base binding; however, this region is not fully conserved in β-tubulins (Burns, 1991). The regions ^{140}GGGTGSG^{146} and ^{178}TVVE^{180}, which are highly conserved in all tubulins, have also been identified as being involved with phosphate and ribose binding, respectively (Mandelkow et al., 1988).

Sternlicht et al. (1987) proposed that the tubulins may resemble other GTPase superfamily members if the unusual step were taken of reversing the orientation of the superfamily nucleotide-binding consensus motifs. Thus, the region ^{103}KGHYTEG^{109} was proposed to be homologous to the superfamily phosphate-binding motif, GXXXXGK, except that its orientation was reversed in β-tubulin. Similarly, the β-tubulin region ^{295}DAKN^{298} was proposed to be equivalent to the base-binding consensus motif, NKXD, of superfamily members and β-tubulin ^{203}DNEA^{206} to be equivalent to the phosphate-binding motif DXXG/A. By homology with ATPases these authors also identified β-tubulin ^{140}GGGTGSG^{146} as a potential phosphate-binding domain.

Interestingly, the regions identified by this approach are very highly conserved in all β-tubulins (Burns, 1991; Burns et al., 1993), and mutations site-directed to the yeast ^{103}KGHYTEG^{109} peptide affected both the assembly-dependent hydrolysis of GTP and microtubule dynamic properties *in vitro* (Davis et al., 1994). In the present study, we have extended this site-directed mutagenesis of the yeast single-copy β-tubulin gene, *TUB2*, to test whether point mutations in the ^{295}DAKN^{298} region affect the affinity and specificity of β-tubulin for GTP binding. We also examined whether a D203S mutation in a proposed phosphate-binding motif of β-tubulin (^{203}DNEA^{206}) affected GTP-binding affinity, the microtubule assembly-dependent GTPase activity, and the dynamic properties of microtubules *in vitro*. The data also provide a comparison to the results of a mutagenesis study by Farr and Sternlicht (1992), who used an unusual method to assay the effects of mutations in these sequences.

MATERIALS AND METHODS

Yeast and Bacterial Strains. The yeast strains used in this study are listed in Table 1. Construction of the mutant strains is described below. The growth conditions of the yeast strains, as well as those of *Escherichia coli*, have been described previously (Sage et al., 1995).

Site-Directed Mutagenesis of TUB2. All mutations were created by the method of Kunkel et al. (1987) using mutagenic oligonucleotides on copies of *TUB2* in the vector pCS3 (Sage et al., 1995). The mutated copies of *TUB2* were introduced into yeast strains carrying the *tub2-590* version of the β-tubulin gene, in which the coding sequence for the C-terminal 12 amino acids had been deleted (Katz & Solomon, 1988). Expression of full-length copies of the β-tubulin gene (mutated or wild type as appropriate) in the *tub2-590* background and the incorporation of the β-tubulins into microtubules were followed immunologically with a rabbit polyclonal antibody specific for the C-terminal 11 amino acids (termed "anti-tail").

For mutations *tub2-D203S*, *tub2-N298K*, and *tub2-N298Q*, fragment-mediated gene replacement of genomic *TUB2* was carried out using 4.9 kb *Sac*I−*Sph*I fragments of mutagenized pCS3. The fragments were transformed into the homozygous *tub2-590/tub2-590* diploid strain ADY101 (Table 1) by the method of Ito et al. (1983). Transformants were selected on the basis of conversion to uracil prototrophy and were screened for the presence of the mutant sequence by polymerase chain reaction (PCR) sequencing of genomic DNA isolated from the transformants. For unknown reasons, the mutation *tub2-D295N* could not be obtained by fragment-mediated transplacement. Instead, *tub2-D295N* was introduced into a hemizygote strain derived from ADY101 (CSY3, *tub2-590*/null, Table 1) on pCS3. This plasmid carries a *CENIII* sequence which maintains the plasmid episomally at a copy number of approximately one per cell (Fitzgerald-Hayes et al., 1982; Sage et al., 1995) and thus effectively regenerated a heterozygous condition in CSY3. Transformants were again selected on the basis of uracil prototrophy, and the presence of the *tub2-D295N* mutation was confirmed by sequencing plasmids "rescued" from candidate yeast strains (Sage et al., 1995).

To determine whether the tubulin mutations introduced into ADY101 by gene replacement were haploid viable, the heterozygous diploids were sporulated (Kassir & Simchen, 1991) and the tetrads dissected using a Lawrence Precision micromanipulator on an Olympus BHC phase contrast microscope. Tetrads from the *tub2-N298K*- and *tub2-*

N298Q-containing heterozygotes produced four viable spores, and haploid strains carrying only the mutated copy of *TUB2* were first identified on the basis of uracil prototrophy. The presence of the mutations in these haploid strains was confirmed by PCR sequencing of genomic DNA.

In tetrads from *tub2-D203S*-containing heterozygotes only two viable spores were produced, and none carried the *URA3* marker cloned into the *TUB2* downstream-flanking sequences. This indicated that the *tub2-D203S* mutation was lethal in haploids, and analysis of this mutation was carried out in the *tub2-D203S/tub2-590* heterozygote.

Isolation of Yeast Tubulin. Tubulin was isolated from 60 L fermenter (Fermicell 130) cultures of yeast strains by the method of Davis et al. (1993). The purity of the isolated tubulins was determined by scanning Coomassie blue stained SDS−PAGE[1] gels of tubulin preparations using a LKB Ultroscan densitometer. The final purities of the tubulin preparations were approximately 80−95%, with the exception of the D295N tubulin which could only be purified to ca. 35%.

In the cases of the D203S and D295N mutations, which we obtained only as heterozygous *tub2-D203S/tub2-590* and *tub2-D295N/tub2-590* diploids, the tubulin preparations were mixtures containing both full-length (*tub2-D203S*, *tub2-D295N*) and truncated (*tub2-590*) β-subunits. Quantitative analysis of Coomassie blue stained SDS−PAGE gels showed that the full-length mutant β-tubulins were present at levels similar to full-length wild-type β-tubulin in a *TUB2/tub2-590* control strain (data not shown).

Determination of Nucleotide-Binding Affinity. GTP-binding assays for yeast tubulins were performed by the method of Hummel and Dryer (1962) as modified for tubulin by Levi et al. (1974), using 12 cm × 0.5 cm columns of P-10 (Bio-Rad, Richmond, CA). The columns were equilibrated in 0.1 M Pipes, pH 6.8, 2 mM EGTA, 1 mM $MgSO_4$ (PEM), 10% (v/v) glycerol, and 5−10 000 nM [^{3}H]GTP (110 Ci mol^{-1}, NEN). Tubulin samples (40−80 μg) in <200 μL volumes were loaded onto the preequilibrated columns at 4 °C, and 0.5 mL fractions were collected. The radiolabel in each fraction was determined by scintillation counting [Beckman LS8000, ReadyProtein (Beckman) scintillant], and the protein content was determined by Bradford assay (Bradford, 1976). This method was also used to analyze binding of radiolabeled XTP (preparation described below) or [γ-^{32}P]ATP (6000 Ci mmol^{-1}, NEN) to tubulins. The binding of GTP to tubulin in the absence of magnesium was also carried out similarly using 12 × 0.5 cm P-10 columns equilibrated with 0.1 M Pipes, pH 6.8, 2 mM EGTA, 5 mM EDTA, 10% (v/v) glycerol, and [^{3}H]GTP over the same concentration range as used above for binding in the presence of magnesium.

Binding constants were obtained from the abscissa intercepts of linear regression lines drawn through Lineweaver−Burk plots, while the binding stoichiometries were obtained from the ordinate intercepts. The results from a representative GTP-binding experiment for each mutant analyzed are shown in Figure 2.

Preparation and Purification of [γ-^{32}P]XTP. [γ-^{32}P]XTP was prepared enzymatically from XDP (Sigma Chemical Co.) and γ-^{32}P]ATP (6000 Ci mmol^{-1}, NEN), using yeast nucleoside diphosphate kinase (Sigma) and reaction conditions described in Garces and Cleland (1969). Radiolabeled XTP was separated from radiolabeled ATP, inorganic phosphate, and XDP using thin-layer chromatography on Whatman paper and a solvent of isobutyric acid, 1 N NH_4-OH, and 0.1 M EDTA (50:30:0.8), as described previously (Zweig & Whitaker, 1971). The specific activity of the radiolabeled XTP was approximately 1500 Ci mol^{-1}.

XTP Competition Assay. The ability of XTP to compete with [^{3}H]GTP for binding to tubulin was measured by incubating tubulin samples (20 μg) with 10 μM [^{3}H]GTP (110 Ci mol^{-1}) and XTP (100 μM−20 mM) in 50 μL reactions in PEM at 4 °C for 30 min. The bound [^{3}H]GTP was separated from unbound radiolabel by spin size exclusion chromatography (3000*g*, 30 s) over a 1 mL G-25 (Sigma) column, previously equilibrated with PEM. The excluded volume containing the tubulin and bound [^{3}H]GTP was assayed to determine the protein concentration (Bradford assay) and radionucleotide content. These values were then used to calculate the stoichiometry of GTP binding to the β-tubulin E-site. The GTP-binding stoichiometry obtained with no XTP present was defined as 100% binding, and the stoichiometries obtained in the presence of XTP were divided by the 100% value to determine the percentage of [^{3}H]GTP remaining bound at the E-site.

Determination of Microtubule Assembly-Dependent GTPase Activity. The assembly-dependent GTPase activity of control microtubules and of microtubules containing D203S mutated tubulin subunits was determined at polymer mass steady state as described in Davis et al. (1993, 1994). All experiments were carried out at 30 °C in the presence of 500 μM [γ-^{32}P]GTP (150−300 Ci mol^{-1} final specific activity). The data were corrected for background (non-assembly-dependent) GTPase activity by subtracting the GTPase activity of tubulin solutions depleted of microtubules by centrifugation. The steady-state data were also corrected for the microtubule number concentration, calculated from the polymer mass at steady state and the average length of the microtubule population (Davis et al., 1993).

Determination of Microtubule Steady-State Dynamic Properties in Vitro. The dynamic properties of individual control microtubules and microtubules containing D203S mutated subunits were examined *in vitro* by VEDIC microscopy, as described by Sage et al. (1995). All experiments were carried out at 30 °C. Determination of the time-averaged growth rates and catastrophic disassembly rates of the microtubules was accomplished as described by Sage et al. (1995).

Immunofluorescence Microscopy. Isolated tubulins were reassembled *in vitro* in PEM plus 1 mM GTP at 30 °C for 30 min. The assembly mix was fixed using 0.5% glutaraldehyde for 5 min at room temperature, placed onto poly(L-lysine)-coated slides, and stained first with the anti-tail rabbit polyclonal antibody specific for the 11 C-terminal amino acids of β-tubulin and susequently with a FITC-conjugated goat anti-rabbit monoclonal antibody (Sage et al., 1995). The microtubules were observed using a Zeiss PMIII fluorescence microscope and a 40× objective. Immunofluorescence

[1] Abbreviations: DAPI, 4,6-diamino-2-phenylindole; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; FITC, fluorescein isothiocyanate; Pipes, 1,4-piperazinediethanesulfonic acid; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; VEDIC, video-enhanced differential interference contrast; XTP, xanthosine 5′-triphosphate.

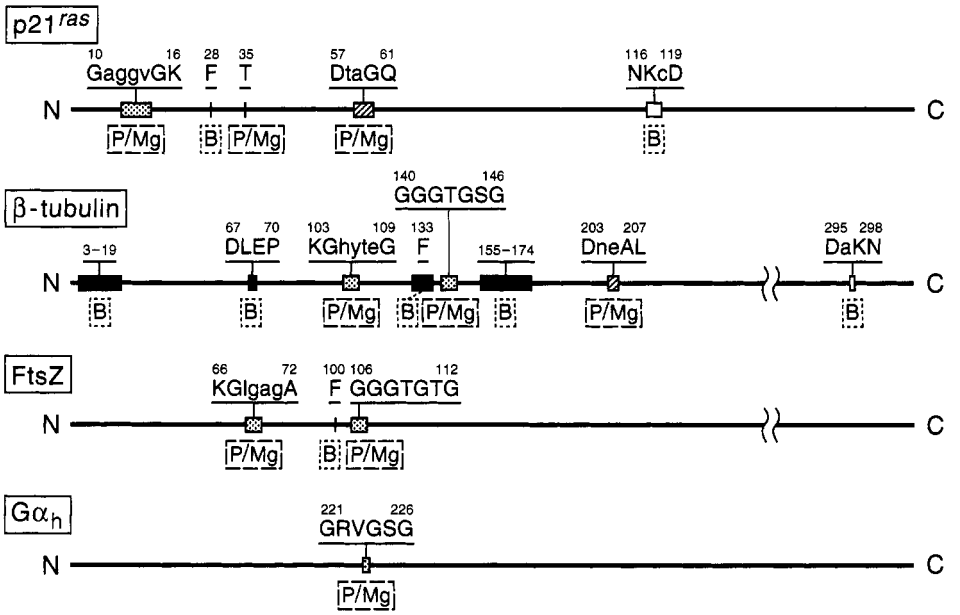

FIGURE 1: Comparison of GTP-binding motifs of the "typical" GTPase superfamily member, p21^{ras}, with those proposed for the "atypical" GTPases β-tubulin, FtsZ, and Gα_h. The principal regions of p21^{ras} interacting with the guanine base (B) or phosphate groups (P/Mg) are shown. X-ray crystallographic studies have demonstrated that these regions are involved in GTP binding and hydrolysis (see text). The regions shown for β-tubulin, FtsZ, and Gα_h are sites proposed to be involved with nucleotide binding and hydrolysis. The peptide composed of amino acids 3−19 of β-tubulin was identified using covalent cross-linking (Shivanna et al., 1993; Jayaram & Haley, 1994) and was proposed to be involved with guanine base binding. The ^{67}DLEP^{70} peptide is highly conserved in virtually all α-, β-, and γ-tubulins (Burns et al., 1993) and is part of a larger peptide composed of amino acids 63−77 also identified by cross-linking studies (Kim et al., 1987; Linse & Mandelkow, 1988; Chavan et al., 1990). Other cross-linking studies identified the β155−174 peptide (Hesse et al., 1987). Primary sequence comparisons with other nucleotide-binding proteins identified ^{178}TVVE^{181}, ^{240}LRFPG^{244} (not shown), and ^{140}GGGTGSG^{146} (Mandelkow et al., 1985). The ^{103}KGHYTEG^{109}, ^{140}GGGTGSG^{146}, ^{203}DNEA^{206}, and ^{295}DAKN^{298} regions were implicated by Sternlicht et al. (1987) by their putative homology to GTPase superfamily members. The β-tubulin Phe133 and Thr143 may be functionally equivalent to the Phe28 and Thr35, respectively, of p21^{ras}. The regions identified in FtsZ are reviewed in Mukherjee and Lutkenhaus (1994), and Gα_h is described by Nakaoka et al. (1994).

staining of cellular microtubules in yeast cells was carried out as described previously (Sage et al., 1995).

Phenotypic Studies of Tubulin Mutants. Determination of the growth rates and benomyl sensitivities of yeast cells were carried out as described previously (Sage et al., 1995).

RESULTS

GTP-Binding Affinities of Mutated Tubulins. Sternlicht et al. (1987) have proposed that the β-tubulin sequences ^{295}DAKN^{298} and ^{203}DNEA^{206} are functionally equivalent to the consensus motifs NKXD and DXXG/A, respectively, of GTPase superfamily members, even though in β-tubulin the DAKN sequence is the reverse of the consensus motif (Figure 1). In the cases of p21^{ras}, EF-Tu, and transducin, crystallographic data have confirmed that the NKXD consensus motif is involved in binding the guanine nucleotide base (Jurnak, 1985; Pai et al., 1990; Noel et al., 1993), and mutagenesis studies with other superfamily members are consistent with these motifs playing a similar role in other superfamily members [reviewed in Bourne et al. (1991) and Valencia et al. (1991)].

To investigate whether mutations in the β-tubulin sequences affected nucleotide binding and hydrolysis similarly to corresponding mutations in the consensus motifs of superfamily members, we introduced mutations into the single-copy β-tubulin gene *TUB2*, transformed the mutated copies into strain ADY101 (Table 1), and isolated wild-type and mutated tubulins from the yeast strains (Davis et al., 1993). The binding affinities of the tubulins for Mg•[^{3}H]-GTP were measured by the equilibrium method of Hummel and Dreyer (1962; Materials and Methods). On the basis of mutagenesis studies with typical superfamily GTPases, the predicted effects of the β-tubulin mutations on nucleotide-binding affinity and specificity are given in Table 2.

Wild-type β-tubulin isolated from the *TUB2/TUB2* diploid strain ADY103 bound Mg•GTP with the same affinity as a control tubulin preparation isolated from a *TUB2/tub2-590* heterozygote (61 ± 12 nM versus 58 ± 7 nM, respectively; Table 2), showing that truncation of the 12 C-terminal amino acids of β-tubulin did not affect Mg•GTP binding. For comparison, phosphocellulose-purified bovine brain tubulin bound Mg•GTP with a slightly lower affinity of 128 ± 9 nM under identical conditions (Figure 2; Table 2).

Since the presence of N298K and N298Q β-tubulin point mutations were not lethal in haploids, we were able to determine the Mg•GTP binding affinities of these mutant tubulins directly in isotypically pure tubulin solutions. Neither mutation significantly altered the binding affinity of tubulin for Mg•GTP: *tub2-N298K* tubulin exhibited a binding affinity of 90 ± 66 nM and *tub2-N298Q* tubulin an affinity of 29 ± 16 nM (Figure 2; Table 2).

For the D295N mutation, which we were unable to obtain in a haploid, and the haploid-lethal D203S mutation, we determined nucleotide binding affinities in tubulin mixtures isolated from heterozygotes. Quantitative densitometry of Coomassie blue stained SDS−PAGE gels showed that the levels of both *tub2-D203S* and *tub2-D295N* were comparable with the *TUB2* level in a *TUB2/tub2-590* control strain. Again, neither mutation had a significant effect on the Mg•GTP binding affinities of β-tubulin: the *tub2-D295N/*

Table 2: Nucleotide Binding Properties of Mutated Yeast Tubulins[a]

tubulin source	K_D (GTP + Mg^{2+}) (nM)	K_D (GTP − Mg^{2+}) (nM)	K_D (XTP + Mg^{2+}) (nM)	K_i (XTP + Mg^{2+}) (mM)	ν (mol/mol)	predicted effect based on $p21^{ras}$
controls						
bovine brain PC	128 ± 9	>500		6	0.4 ± 0.1	
TUB2/TUB2	61 ± 12	>500	>500	6	0.9 ± 0.2	
TUB2/tub2-590[b]	58 ± 7				1.0 ± 0.1	
^{295}DAKN^{298}						
tub2-D295N/tub2-590	46 ± 3	>500	>500	5	0.5 ± 0.1	favor XTP binding
tub2-N298K	90 ± 66				0.8 ± 0.4	reduce Mg•GTP binding
tub2-N298Q	29 ± 16				0.4 ± 0.1	reduce Mg•GTP binding
^{203}DNEA^{206}						
tub2-D203S/tub2-590	43 ± 6	>500			0.8 ± 0.2	reduce Mg•GTP binding

[a] Equilibrium dissociation constants for nucleotide binding were determined using the method of Hummel and Dreyer (1962) and were obtained from the abscissa intercepts of double-reciprocal plots of the data, using linear regression analysis. The maximum stoichiometries of GTP binding (ν, moles of GTP bound per mole of tubulin dimer) were obtained from the ordinate intercepts of the double-reciprocal plots. Each value is the average of at least three experiments, and errors represents ±1 standard error of the mean. The predicted effects of mutations on tubulin−Mg•GTP binding are based on the results of mutagenesis studies with GTPase superfamily members, which are described in the text. [b] From Davis et al. (1993).

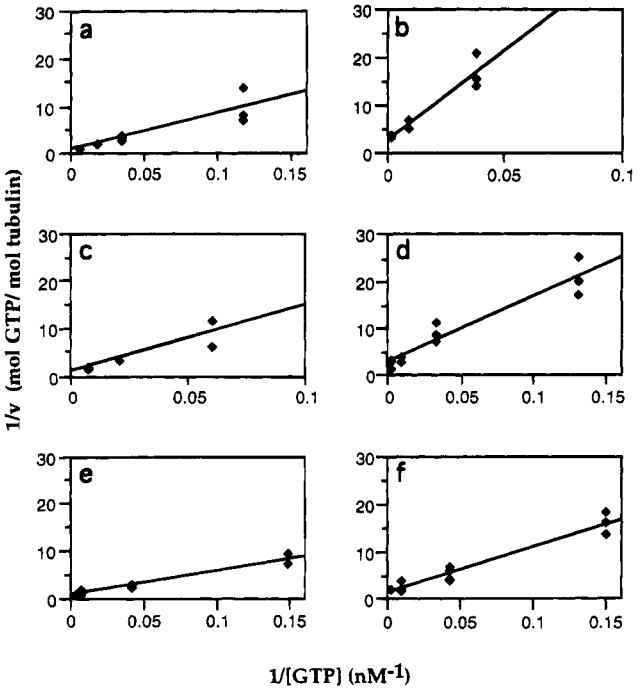

FIGURE 2: GTP binding to tubulins isolated from β-tubulin mutants. The different tubulin preparations (40−80 μg in <200 μL volumes) were incubated at 4 °C with 5−10 000 nM [^{3}H]GTP for 30 min, and Mg•GTP binding to the tubulins was determined by the method of Hummel and Dreyer (1962), as described in Materials and Methods. The equilibrium binding constants were obtained from double-reciprocal plots of the binding data using linear regression analysis. The tubulins were isolated from (a) a *TUB2/tub2-590* yeast heterozygous diploid control, (b) bovine brain by three cycles of assembly−disassembly and phosphocellulose chromatography, (c) a *tub2-N298K* haploid, (d) a *tub2-N298Q* haploid, (e) a *tub2-D203S/tub2-590* heterozygous diploid, and (f) a *tub2-D295N/tub2-590* heterozygous diploid.

tub2-590 tubulin mixture bound Mg•GTP with an affinity of 46 ± 3 nM and the *tub2-D203S/tub2-590* mixture with an affinity of 43 ± 6 nM (Figure 2; Table 2).

To examine whether the method of Hummel and Dreyer (1962) for determining GTP binding behaved similarly to previously used methods, we also measured GTP binding to tubulins in the absence of Mg^{2+}. Previously, it had been shown that the affinity of vertebrate brain tubulin for GTP was reduced 160−1100-fold in the absence of Mg^{2+} [*e.g.*, Correia et al. (1987) and Mejillano and Himes (1991)] and that the GTP binding to some mutated β-tubulins was Mg^{2+}-sensitive (Farr et al., 1992).

In the absence of Mg^{2+}, no GTP binding was detected to bovine brain tubulin, to yeast control tubulin, or to D295N and D203S mutated yeast tubulins, even with concentrations as high as 500 nM GTP. Because of the signal-to-noise limitations of the method of Hummel and Dreyer (1962) this was the highest GTP concentration experimentally feasible. The K_D for GTP binding in the absence of Mg^{2+} is therefore greater than 500 nM for each of these tubulins, and both control and mutated tubulins therefore retained a requirement for Mg^{2+} in GTP binding (Table 2).

Nucleotide-Binding Specificity of the D295N Mutation. A main feature of the proposal (Sternlicht et al., 1987) that the β-tubulin ^{295}DAKN^{298} sequence is cognate to the superfamily NKXD motif is that the aspartate residue should determine nucleotide-binding specificity. In $p21^{ras}$ and EF-Tu substituting asparagine for the aspartate in the NKXD motif both reduced the binding affinity of these proteins for GTP and changed the nucleotide-binding specificity to XTP or ITP (Hwang, 1987; Sigal, 1986; Weijland & Parmeggiani, 1993). We therefore investigated directly whether Mg•[γ-^{32}P]XTP would bind to the *tub2-D295N/tub2-590* tubulin mixture using the method of Hummel and Dreyer (1962). For comparison, we also examined radiolabeled XTP binding to control tubulin (*TUB2/tub2-590*) preparations. In neither case was Mg•XTP binding observed, up to a concentration of 500 nM (Table 2). This placed a lower limit of 500 nM on the K_D for XTP binding to the mutated and control tubulins.

To investigate whether XTP would bind to the mutated tubulin at higher nucleotide concentrations than were possible with the Hummel−Dreyer method, we examined the ability of XTP to compete with [^{3}H]GTP for binding to the *tub2-D295N/tub2-590* mixture. For comparison, we also examined XTP inhibition of radiolabeled GTP binding to control and bovine brain tubulins. As shown in Figure 3, the XTP competed similarly for GTP binding to all tubulin samples, and the apparent inhibition constants of XTP for GTP binding were essentially the same (6 mM for bovine brain and control yeast tubulins, 5 mM for the *tub2-D295N/tub2-590* mixture; Table 2).

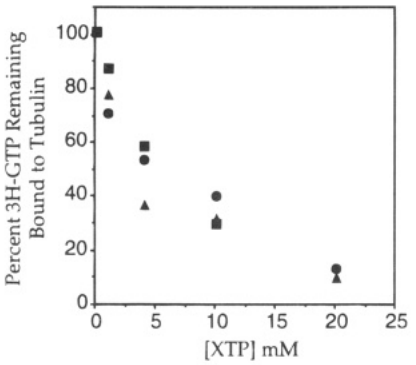

FIGURE 3: Competition of XTP for GTP binding to tubulin. The ability of XTP to compete with [^{3}H]GTP for binding to bovine brain phosphocellulose-purified tubulin (squares), *TUB2*/TUB2 wild-type yeast tubulin (circles), and *tub2-D295N*/*tub2-590* heterozygote tubulin (triangles) was performed at 4 °C. Tubulin samples (20 μg) were incubated with 10 μM [^{3}H]GTP and from 100 μM to 20 mM XTP for 30 min, and the extent of GTP binding to the tubulin preparations was determined chromatographically (Materials and Methods). Maximal GTP binding (100%) was defined as the amount of GTP that bound to a tubulin preparation in the absence of XTP.

Finally, we also used the Hummel−Dreyer method to determine whether D295N tubulin had an altered affinity for ATP binding. At 500 nM Mg^{2+}·[γ-^{32}P]ATP both control and the mutated tubulin mixtures showed very low stoichiometries of binding, indicating that both tubulin samples had a similar and weak affinity for ATP binding (data not shown). Thus, the D295N point mutation also does not appear to alter the affinity of β-tubulin for ATP.

A potentially trivial explanation for the lack of effect of the D203S and D295N mutations on nucleotide binding is that the mutated tubulins might simply be inactive when isolated and that the binding assays merely detect nucleotide binding to the truncated form of wild-type β-tubulin (*tub2-590*) in the mixtures. Immunofluorescence analysis of microtubules in the heterozygous strains carrying these mutations, using the anti-tail antibody specific for the C-terminal amino acids of the mutated β-tubulins [e.g., Davis et al. (1994) and Sage et al. (1995)], indicated that the mutated β-tubulins assembled into all cellular microtubules (Figure 4a−j). Nevertheless, the possibility still remained that the mutant tubulins were more readily inactivated during the isolation procedure, even though analysis by SDS−PAGE did not indicate increased proteolytic degradation of the mutated proteins.

We therefore tested the ability of the mutated β-tubulins to assemble into microtubules *in vitro*. Mixtures of the *tub2-D203S*/*tub2-590* and *tub2-D295N*/*tub2-590* tubulins were assembled *in vitro* and the microtubules fixed and stained for immunofluorescence microscopy using the anti-tail antibody. The results showed that microtubules reassembled from both tubulin mixtures exhibited good immunofluorescence staining, showing that the mutated tubulins were assembly-competent *in vitro* (Figure 4k,l). Thus, it seems unlikely that the mutated subunits are inactive *in vitro* and are incapable of binding nucleotide.

Effects of the D203S Mutation on Microtubule Assembly-Dependent GTPase Activity and Dynamics in Vitro. Because mutations putatively cognate to the D203S β-tubulin mutation decreased the GTPase activity of superfamily members, we examined the effects of this mutation both on the assembly-dependent GTPase activity of microtubules and on the dynamic properties of individual microtubules *in vitro* (Table 3).

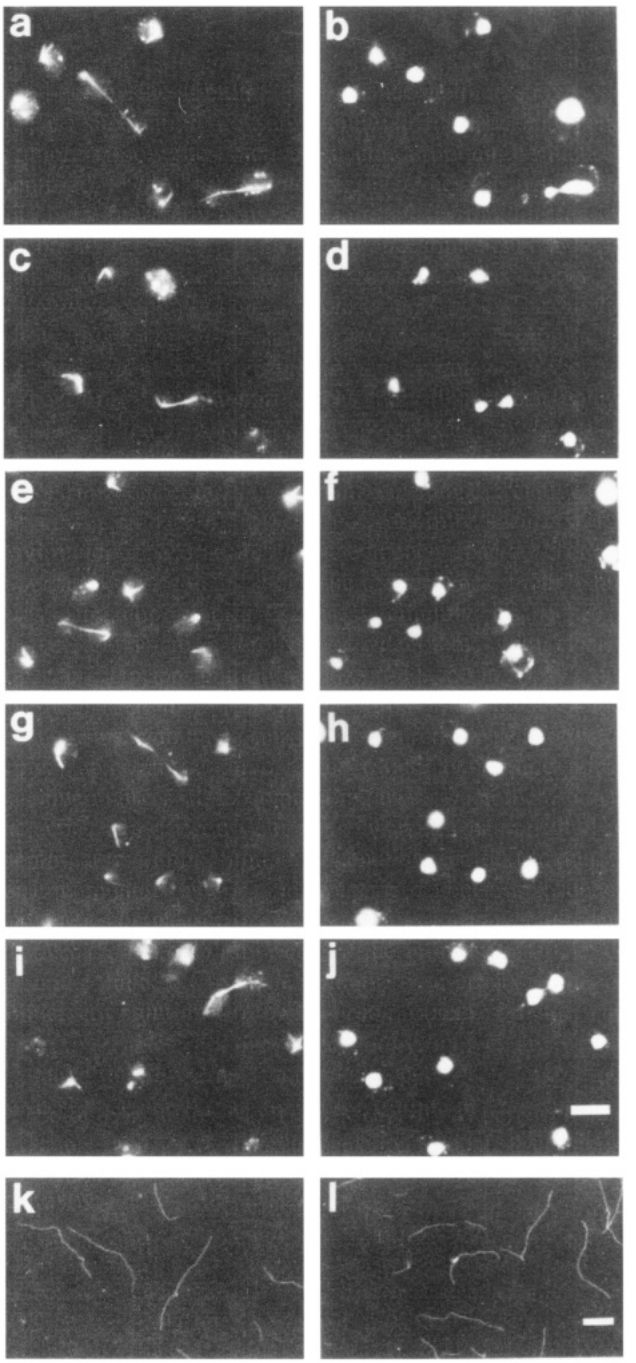

FIGURE 4: Incorporation of mutated tubulins into yeast microtubules *in vivo* and *in vitro*. (a, c, e, g, i) Incorporation of wild-type or mutated full-length β-tubulins into cellular microtubules of log-phase yeast cultures was detected using the anti-tail antibody (Materials & Methods). (b, d, f, h, j) Nuclear DNA was localized using DAPI staining: (a, b) *TUB2*/*TUB2* homozygous diploid; (c, d) *tub2-N298K* haploid; (e, f) *tub2-N298Q* haploid; (g, h) *tub2-D203S*/*tub2-590* heterozygous diploid; (i, j) *tub2-D295N*/*tub2-590* heterozygous diploid. Incorporation of mutated β-subunits into microtubules assembled *in vitro* from tubulin isolated from (k) a *tub2-D203S*/*tub2-590* heterozygote or (l) a *tub2-D295N*/*tub2-590* heterozygote was followed by immunofluorescence microscopy using the anti-tail antibody (Materials & Methods). Bar = 5 μm.

Tubulin samples for *in vitro* reassembly were isolated from the *tub2-D203S*/*tub2-590* heterozygote or from *TUB2*/*tub2-590* control cells. The solutions of tubulin α/β dimers were therefore composed of mixtures containing full-length wild-

Table 3: Effects of the D203S Mutation on the Assembly-Dependent GTPase Activity and Dynamic Properties of Steady-State Microtubules[a]

tubulin source	microtubule GTPase activity (P_i min^{-1} microtubule^{-1})	time-averaged growth rate at microbutule (+)-ends (μm h^{-1})	catastrophic disassembly rate (s^{-1})
TUB2/tub2-590	900 ± 640	17 ± 1	3000 ± 300
tub2-D203S/tub2-590	1700 ± 1600	20 ± 1	2600 ± 1000

[a] The microtubule GTPase activity was determined at the polymer mass steady state, as described in Davis et al. (1993, 1994), and has been corrected for nonassembly-dependent GTPase activity and the concentration of microtubule ends. The values are the average of four separate determinations. The time-averaged growth rate and catastrophic disassembly rate were determined at steady state for microtubule (+)-ends, as described in Sage et al. (1995). The values were obtained from observing 20 different microtubules. All experiments were carried out at 30 °C. Errors represent ±1 standard error of the mean.

type β-tubulin and truncated *tub2-590* β-tubulin (control sample) or full-length mutated D203S β-tubulin and truncated *tub2-590* β-tubulin (experimental sample). The full-length and truncated β-tubulins were present in equimolar proportions in the mixtures (data not shown). The tubulin samples were assembled to polymer mass steady state in the presence of 500 μM [γ-^{32}P]GTP, and at steady state the rate of GTP hydrolysis was determined from the rate of evolution of radiolabeled inorganic phosphate. The data were corrected for background (non-assembly-dependent) GTPase activity and for the microtubule number concentration (Materials and Methods).

As shown in Table 3, the mean value for the assembly-dependent steady-state GTPase activity of microtubules containing D203S β-tubulin was somewhat greater than that of control microtubules. The average values from four separate experiments were 1700 ± 1600 P_i min^{-1} microtubule^{-1} for D203S-containing microtubules versus 900 ± 600 P_i min^{-1} microtubule^{-1} for control microtubules. However, because of the large variation between experimental determinations, these differences are not statistically significant.

The effects of the D203S mutation on the dynamic properties of (+)-ends of individual microtubules were also determined, using VEDIC microscopy (Table 3). The tubulin preparations were again isolated from the *tub2-D203S/tub2-590* and *TUB2/tub2-590* heterozygotes and reassembled to steady state at 30 °C in 500 μM GTP.

As observed previously (Davis et al., 1993; Sage, et al., 1995) both control and mutant individual yeast microtubules were far less dynamic than vertebrate brain microtubules *in vitro* and did not exhibit the large and frequent growing and shortening length changes characteristic of vertebrate microtubules (data not shown). Instead, the yeast microtubules continually grew until a rapid shortening event occurred. "Rescues", in which a shortening microtubule began regrowing, were rarely seen with either control or mutant microtubules; consequently, when a disassembly event occurred, both types of microtubules usually disassembled completely. As a consequence of these unusual dynamic properties of yeast microtubules, we measured only the time-averaged growth rate of the microtubules over the lifetime of the microtubule and the catastrophic disassembly rate. All measurements were carried out for the (+)-ends of microtubules as described by Sage et al. (1995).

The time-averaged growth rate at (+)-ends of steady-state microtubules containing D203S β-tubulin subunits was slightly faster (20 ± 1 μm h^{-1}) than that of control microtubules (17 ± 1 μm h^{-1}). The growth rate of the control microtubules was essentially identical to that measured previously for control microtubules (Sage et al., 1995). In contrast, there was essentially no difference in the catastrophic disassembly rates of the "mutant" and control microtubules (2600 ± 1000 s^{-1} versus 3000 ± 300 s^{-1} for control microtubules; Table 3).

Phenotypic Effects of the β-Tubulin Mutations. Phenotypic analyses of the β-tubulin mutations demonstrated that all but the D203S mutation had minor effects and were generally consistent with the lack of effects of the mutations on nucleotide binding and microtubule assembly. None of the mutations affected the cell-doubling times at normal growth temperatures nor produced temperature-sensitive growth at either 37 or 13 °C (Table 4). Most of the mutations did affect the sensitivity of cell growth to benomyl but fell within only a 2-fold increase in drug sensitivity. The sole exception was the N298Q mutation, which had no effect on benomyl sensitivity. The most severe phenotypic effects was the haploid lethality of the D203S mutation (Table 4).

DISCUSSION

The tubulin family of proteins, the α-, β-, and γ-tubulins, are atypical members of the GTPase superfamily. None shows the superfamily arrangement of consensus motifs found in virtually all other members, and only β-tubulin has been shown to hydrolyze GTP. Moreover, β-tubulin binds GTP exchangeably whereas GTP binding to α-tubulin is nonexchangeable. Our goal has been to identify β-tubulin sites that interact with the bound nucleotide and participate in nucleotide hydrolysis. We hope to understand whether the different nucleotide-binding and hydrolyzing properties of the α-, β-, and γ-tubulins, as well as their different cellular functions, are related to changes in their nucleotide-binding sequences.

We have used site-directed mutagenesis to test part of a model for the exchangeable GTP-binding site of β-tubulin. Specifically, we examined whether the β-tubulin sequences ^{203}DNEA^{206} and ^{295}DAKN^{298} participate in binding GTP, as suggested by Sternlicht et al. (1987; Figure 1). These authors proposed that the ^{295}DAKN^{298} motif is equivalent to the NKXD consensus motif of typical GTPase superfamily members, even though in β-tubulin the sequence is reversed. Although it is difficult to see how the β-tubulin sequence could function equivalently, other examples of consensus motifs functioning in either forward or reverse orientation are known (Chiang & Dice, 1988; Becker & Roth, 1992). Indeed, mutations in the β-tubulin sequence ^{103}KGHYTEG^{109}, which Sternlicht et al. (1987) proposed is the reversed equivalent to the GXXXXGK phosphate-binding motif of superfamily members, affect the assembly-dependent hydrolysis of GTP by tubulin (Davis et al., 1994). There are also a number of examples, including members of the GTPase superfamily, in which proteins with apparently unrelated primary sequences have similar tertiary structures (Bourne et al., 1991; Flaherty et al., 1991) or similar conjunctions of active site groups (Pearl, 1993).

In p21^{ras} and EF-Tu the NKXD motif plays a primary role in binding the nucleotide base, and mutations in this sequence

Table 4: Phenotypic Analysis of Proposed β-Tubulin GTP-Binding Mutants[a]

yeast strain	β-tubulin genotype	growth rate at 30 °C (h)	benomyl sensitivity (μg/mL)	haploid viability	growth at 37 °C	growth at 13 °C
ADY103	*TUB2/TUB2*	2.0 ± 0.1	48	yes	+	+
CSY3-TUB2	*tub2-590/TUB2*	2.0 ± 0.1	24	NA[b]	+	+
CSY3-D295N	*tub2-590/tub2-D295N*	1.9 ± 0.1	10	*c*	+	+
D203S	*tub2-590/tub2-D203S*	2.2 ± 0.1	10	no	+	+
FY41	*TUB2*	2.2 ± 0.1	40	yes	+	+
N298K	*tub2-N298K*	2.3 ± 0.1	20	yes	+	+
N298Q	*tub2-N298Q*	2.1 ± 0.1	40	yes	+	+

[a] Doubling times were determined in supplemented SD media (Materials and Methods) and are the average values of four to six separate determinations ±1 standard error of the mean. Benomyl and temperature sensitivities of strains were determined on SD agar plates and examined after 3 days of growth (14 days for 13 °C growth). The haploid viability of spores was determined by tetrad dissection and growth of the spores on supplemented SD at 30 °C. Spore viability was assessed visually or microscopically after 3−5 days. [b] NA = not applicable. [c] It was not possible to obtain *tub2-D295N* as a heterozygote by genomic replacement.

greatly reduce nucleotide binding (Der et al., 1986; Feig et al., 1986; Sigal et al., 1986). Moreover, substitution of Asn for Asp138 in EF-Tu (Hwang, 1987) or Ala for Asp119 in $p21^{ras}$ (Sigal, 1986) changed the nucleotide specificity of the proteins from guanine to xanthine and inosine, respectively. Thus, a critical test of the model of Sternlicht et al. (1987) is that a D295N mutation should reduce the GTP-binding affinity of β-tubulin and change the nucleotide-binding specificity from GTP to XTP. However, tubulin isolated from a *tub2-D295N/tub2-590* yeast strain had an affinity for GTP that was essentially the same as wild type (K_D = 46 ± 3 nM versus 61 ± 12 nM for wild type; Figure 2, Table 2). Moreover, the affinity of this tubulin mixture for XTP was not increased over that of wild-type yeast tubulin, and the inhibition constant for XTP inhibition of GTP binding also was essentially the same as that of wild-type yeast and bovine brain tubulins (Table 2; Figure 3). Clearly, the absence of an effect of the D295N mutation on either GTP-binding affinity or nucleotide-binding specificity could be due to an artifact caused by the underexpression or inactivation of the mutated subunit in the tubulin mixture. This is unlikely, however, since D295N β-tubulin is present at levels equivalent to that of wild-type β-tubulin (data not shown) and was assembly-competent both in cells and *in vitro* (Figure 4i,l). The lack of effect of this mutation on GTP binding and specificity is in marked contrast to the effects of a D138N mutation in the NKXD motif of EF-Tu, for example, where XTP bound to the mutated EF-Tu with an affinity comparable to that for GTP binding to wild-type EF-Tu (Hwang & Miller, 1987).

These results were complemented by the lack of effect of the *tub2-N298K* and *tub2-N298Q* mutations on GTP binding. With K_D's of 90 ± 66 nM and 29 ± 16 nM, respectively, the affinities of these β-tubulins for GTP were not markedly different from that of wild-type β-tubulin (61 ± 12 nM). For comparison, substituting Lys for Asn116 in the $p21^{ras}$ NKXD motif completely abolished the GTP-binding activity of the protein (Clanton, 1986). Furthermore, the growth rates of *tub2-N298K* and *tub2-N298Q* haploid cells were unaffected, and the strains had benomyl sensitivities close to those of wild-type cultures (Table 4). The normal GTP-binding affinities of the N298K and N298Q mutations, and the lack of effect of the D295N mutation on nucleotide-binding specificity (Table 2), demonstrate that mutations in the ^{295}DAKN^{298} sequence do not affect GTP-binding equivalently to mutations in the NKXD consensus motif of conventional GTPases.

Sternlicht et al. (1987) also proposed that the Asp203 of β-tubulin is cognate to the aspartate in the GTPase superfamily consensus motif DXXG. In the crystal structures of $p21^{ras}$ and EF-Tu complexed with the GTP analogue guanosine 5′-(β,γ-imido)triphosphate the putatively cognate aspartate residues are coordinated to Mg^{2+} via a water molecule (Pai et al., 1990; Berchtold et al., 1993). The Mg^{2+} also coordinates with the β,γ-phosphates of the bound nucleotide, and a change in Mg^{2+} coordination upon GTP hydrolysis appears to trigger large conformational changes between the GTP- and GDP-bound forms of the proteins (Schlichting et al., 1990). It is not surprising therefore that Mg^{2+} is an important cofactor in GTP binding for GTPase superfamily members (Gilman, 1987) and that mutations in the DXXG superfamily motif reduce the affinity for Mg•GTP binding and impair GTP hydrolysis (John et al., 1993).

On the basis of the structures of $p21^{ras}$ and EF-Tu, it would be expected that substituting a serine for the β-tubulin Asp203 would remove the ability of this residue to act as a hydrogen acceptor for a water molecule, thereby impairing coordination of the Mg^{2+} and disrupting proper nucleotide binding and hydrolysis. Experimentally, however, tubulin isolated from the *tub2-D203S/tub2-590* mutant exhibited essentially the same affinity for GTP as wild-type tubulin (K_D = 43 ± 6 nM versus 61 ± 15 nM, respectively; Figure 2, Table 2), and the *in vitro* assembly-dependent GTPase activity of tubulin from the *tub2-D203S/tub2-590* heterozygote also was not significantly different from control cells (Table 3). For comparison, mutations in the ^{103}KGHYTEG^{109} site of yeast β-tubulin increased the GTPase activity 4−10-fold (Davis et al., 1994), and a T143G mutation in the ^{140}GGGTGSG^{146} β-tubulin sequence reduced the GTPase activity approximately 10-fold (C. Dougherty and K. Farrell, unpublished observations). It is unlikely that the lack of effects of the D203S mutation on β-tubulin GTP binding and hydrolysis is an artifact caused by inactivation of the D203S β-tubulin in the mixture, since the mutant subunits were able to assemble into microtubules both in cells and *in vitro* (Figure 4i,l).

Furthermore, the effects of the D203S mutation on microtubule (+)-end dynamics are difficult to rationalize on the basis of an effect on GTP binding and/or hydrolysis. An impaired tubulin GTPase activity would be expected to lead to superstabilized microtubules containing increased numbers of tubulin−GTP subunits (Mitchison & Kirschner, 1984), yet the catastrophic disassembly rate of microtubules containing D203S subunits is statistically indistinguishable from

those of control microtubules (Table 3). The effects of the D203S mutation, to increase slightly the time-averaged growth rate of microtubule (+)-ends without significantly affecting the microtubule disassembly rate (Table 3), therefore indicate that the β-tubulin Asp203 may play a role in microtubule assembly but probably not via the tubulin−GTP site.

Other considerations also suggest that the β-tubulin ^{203}DNEA^{206} sequence cannot fulfill the same role as the DXXG motif of superfamily members. A comparison of the crystal structures of GDP- and GTP-bound forms of p21^{ras} and EF-Tu shows that the invariant glycine (Gly60 in p21^{ras}, Gly83 in EF-Tu) is part of a region which undergoes a large conformational change between the two forms [e.g. Schlichting et al. (1990)]. The inherent flexibility of the glycine is considered necessary for this conformational change, and this idea is supported by the observation that substituting an alanine for Gly83 in EF-Tu inhibited its GTP-dependent association with aminoacyl-tRNA (Hwang et al., 1989). Given the fact that β-tubulin has an Ala206 rather than Gly206 at the putative cognate position, it seems highly unlikely that the ^{203}DNEA^{206} motif could play a role exactly equivalent to that of the DXXG motif of typical superfamily members.

In a recent study, Farr and Sternlicht (1992) examined the effects of β-tubulin mutations on nucleotide binding to *in vitro* translation products from site-directed mutants of chick β2 mRNA. Nucleotide-binding affinity was assayed from the amount of translation product eluted off an anion-exchange FPLC column at different concentrations of GTP. Although our above conclusions regarding the roles of the ^{203}DNEA^{206} and ^{295}DAKN^{298} sequences in nucleotide binding are generally consistent with those of these authors, there are some notable differences in experimental results. For example, these authors found that a D203N mutation decreased Mg•GTP binding nearly 200-fold without altering the requirement for Mg^{2+}, whereas we found that a D203S substitution had no measurable effect on nucleotide binding, again without altering the requirement for Mg^{2+} (Figure 2; Table 2). There may be many reasons for this difference. For example, the two mutations are structurally different, which may account for the observation that the D203N mutation was unusually sensitive to tryptic digestion and coassembled poorly with brain tubulin (Farr & Sternlicht, 1992), whereas the D203S tubulin assembled well both in cells and *in vitro* (Figure 4). The poor assembly of the D203N mutation and its unusual sensitivity to proteolysis may be indicative of an unstable protein, which could result in an underestimation of its GTP-binding affinity. Regardless of the reason, neither result supports a role for Asp203 in coordinating with the Mg^{2+} moiety in Mg•GTP.

More puzzling, however, is the observation that, in our hands using the method of Hummel and Dreyer (1962), a D224N yeast β-tubulin mutation kindly provided by Dr. Himan Sternlicht increased the binding affinity for Mg•GTP by approximately 5-fold (to a K_D = 9 nM) and retained the requirement for Mg^{2+} (C. R. Sage and K. Farrell, unpublished observation). In contrast, Farr and Sternlicht (1992) using their assay found that this same mutation in chick β-tubulin decreased Mg•GTP binding nearly 100-fold and drastically diminished the requirement for Mg^{2+}. The reason for this difference is unclear. It seems unlikely to be related to phyllogenetic differences in tubulin source, since the binding constant we measured for wild-type yeast tubulin is similar to that for bovine brain tubulin (Table 2) and falls within the range of values observed by other researchers [*e.g.*, see Mejillano and Himes (1991)]. Another possibility is that the assay method used by Farr and Sternlicht (1992) is sensitive to additional factors which could influence the ability of GTP to elute the β-tubulin transcripts off the anion-exchange column.

Tubulins Are Atypical GTPases. The results of the mutagenesis data argue that the ^{203}DNEA^{206} and ^{295}DAKN^{298} sequences do not function equivalently to the DXXG and NKXD consensus motifs of superfamily members. A caveat to any conclusion based solely on primary sequence mutagenesis, however, is that the relationship between primary sequence and tertiary structure is poorly understood and not always straightforward. A good example of this is the near-superimposable tertiary structures of hsp-70 and actin, despite a mere 17% identity in their primary structures (Flaherty et al., 1991). Moreover, a comparison of the acyl-enzyme intermediates formed by β-lactamase and endothiapepsin indicated that catalytic mechanisms can be conserved even without the conservation of active site residues (Pearl, 1993). However, the data clearly show that the mutations we introduced into the two β-tubulin sequences do not affect tubulin in the same way as mutations in the GTPase superfamily motifs proposed to be cognates. This and the observations that the mutated β-tubulins are assembly competent both in cells and *in vitro* (Figure 4) indicate either that these sites do not contribute to the β-tubulin GTP-binding pocket or that the mechanism of guanine ring binding in β-tubulin is quite different from that of other superfamily members such as p21^{ras} or EF-Tu.

Several studies have used UV cross-linking of GTP or GTP analogues to β-tubulin to identify potential domains involved in binding the guanine moiety (Chavan et al., 1990; Hesse et al., 1987; Jayaram & Haley, 1994; Kim et al., 1987; Linse & Mandelkow, 1988; Shivanna et al., 1993). While it is possible that the sequences identified by these studies could be artifacts caused by UV perturbation of the β-tubulin structure, cross-linking studies using periodate-oxidized GTP correctly identified the NKXD consensus motif in p21^{ras} (Low et al., 1993) and EF-Tu (Peter et al., 1988). The β-tubulin sequences identified by the photoaffinity labeling studies are β9−15 (Jayaram & Haley, 1994; Shivanna et al., 1993), β63−77 (Chavan et al., 1990; Kim et al., 1987; Linse & Mandelkow, 1988), and β155−174 (Hesse et al., 1987).

Of these, almost no homology exists between the β9−15 sequence and that in α-tubulin (Burns et al., 1993), which also is presumed to bind GTP; furthermore, the β-tubulin Cys12, identified as the residue to which GTP covalently cross-linked (Shivanna et al., 1993), is replaced with Tyr12 in the yeast β-tubulin sequence. Thus, this sequence presumably would not have been identified by this approach if yeast tubulin had been used instead to bovine tubulin.

In contrast, β63−77 shows a high degree of conservation in all α- and β-tubulins; the sequence also includes two aspartates (Asp67, Asp74) that are conserved in all β-tubulins and which could act as cognates to the p21^{ras} Asp119 in binding to the guanine ring. Farr and Sternlicht (1992) found that a D67N mutation abolished nucleotide binding to β-tubulin, which would be consistent with this residue interacting with the guanine moiety. However, for reasons discussed above, there is some question as to whether the

assay method used in this study is sensitive only to nucleotide-binding affinity. Also, as the authors themselves pointed out, an alternative possibility is that the primary effect of the mutation could simply be to perturb the protein conformation (Farr & Sternlicht, 1992). In yeast, a D67A substitution did not affect cell viability (Reijo et al., 1994), suggesting that GTP binding might not be affected; however, purely phenotypic screens of mutations may not detect mutants with reduced GTP binding, since the intracellular concentration of GTP could still be sufficiently high to saturate the nucleotide-binding site.

The β155−174 sequence also shows good conservation in β-tubulins and includes a highly conserved Asp161. Furthermore, a D161A mutation in yeast was supersensitive to benomyl, which could indicate a problem in tubulin−nucleotide interactions (Reijo et al., 1994). However, this sequence is poorly conserved in α-tubulins, and the highly conserved Asp161 is replaced by a Lys163 (Burns et al., 1993). If this β-tubulin sequence is involved with binding the guanine moiety, then details of guanine ring binding in α-tubulin must be quite different from those of β-tubulin.

The tubulin family of proteins thus presents an interesting enigma. The data presented in this paper argue that the DNEA and DAKN β-tubulin sequences are not cognates of the GTPase superfamily consensus motifs. Furthermore, photoaffinity-labeling studies identify putative β-tubulin guanine ring binding sequences which show no obvious homologies with superfamily consensus sequences. In contrast, mutations in the ^{103}KGHYTEG^{109} sequence of yeast β-tubulin affect the microtubule-dependent GTPase activity (Davis et al., 1994) and suppress microtubule dynamics *in vitro* (Sage et al., 1995). In addition, a mutation in the putative phosphate-binding motif ^{140}GGGTGSG^{146} both reduces GTP binding and suppresses microtubule dynamics (C. Dougherty and K. Farrell, unpublished observation). These studies are thus consistent with the idea that β-tubulin possesses a cryptic GXXXXGK superfamily motif, as well as a glycine-rich phosphate-binding sequence (^{140}GGGTGSG^{146}) characteristic of many ATPases [*e.g.*, Dreusicke and Schulz (1986)].

A simplistic picture of the β-tubulin GTP site which emerges from these studies is one in which the primary sequences involved in binding the guanine moiety are apparently unrelated to those of "typical" GTPases whereas those that contribute to phosphate binding show good (in the case of ^{140}GGGTGSG^{146}) or cryptic (^{103}KGHYTEG^{109}) homologies with other nucleotide-binding proteins. The order in which the putative nucleotide-binding sequences are arranged along the primary sequence of β-tubulin also differs from that of typical GTPase superfamily members. In p21^{ras}, all primary phosphate-binding sequences (^{10}GAGGVGK^{16}, Thr35, ^{57}DTAG^{60}) are N-terminal to the primary base-binding sequence (^{116}GAGGVGK^{16}, Thr35, ^{57}DTAG^{60}) are N-terminal to the primary base-binding sequence (^{116}NKCD^{119}), an arrangement common to the GTPase superfamily (Bourne et al., 1991). In β-tubulin, by contrast, the putative phosphate-binding sequences (β103−109, β140−146) are C-terminal to two of three base-binding sequences identified by photoaffinity labeling (β9−15, β63−77); only one (β155−174) is C-terminal to the putative phosphate-binding sequences.

Taking these considerations at face value, it appears that the topology of the GTP-binding pocket of β-tubulin differs significantly from those of typical GTPases. Presumably, the same also is true of α- and γ-tubulins, the other known members of the tubulin family, since neither shows the GTPase superfamily consensus motifs. Instead, the α- and γ-tubulins show the putative nucleotide-binding sequences identified for β-tubulin. However, even these sequences are not always perfectly conserved in α- and γ-tubulins, and it has been suggested that such differences may contribute to the differing nucleotide binding and hydrolysis properties of the α-, β-, and γ-tubulins (Burns et al., 1993). The tubulin family of proteins thus appears to represent examples of "atypical" GTPases. Recently, other examples of *bona fide* GTPases have been reported that show imperfect (IAP86; Kessler et al., 1994), little (FtsZ; de Boer et al., 1992; RayChaudhuri & Park, 1992), or no ($G\alpha_h$; Nakaoka et al., 1994, Takeuchi et al., 1992) primary sequence relationship to typical members of the GTPase superfamily, such as p21^{ras} (Figure 1). There is thus good evidence that the GTPase superfamily of proteins should be broadened to include not just the families of typical GTPases exemplified by the p21^{ras}, EF-Tu, and small heterotrimeric G-proteins with clearly conserved consensus motifs (GxxxxGK, T, DxxG, NKxD) but should also include atypical families of proteins such as the tubulins, which do not show the consensus motifs of typical superfamily members and which also do not appear to show obvious sequence homologies between themselves. Sequence comparison of FtsZ to β-tubulin has resulted in identification of only limited sequence similarity (Mukherjee & Lutkenhaus, 1994), and we uncovered no sequence similarities between $G\alpha_h$ and β-tubulin or between $G\alpha_h$ and FtsZ. Atypical GTPases such as these, as well as the α-, β-, and γ-tubulins, thus most likely represent examples of convergent solutions to the GTPase problem.

ACKNOWLEDGMENT

The authors thank Mr. Herb Miller for expert technical assistance and Dr. Frances Jurnak for critical discussions.

REFERENCES

Baker, P. J., Britton, K. L., Rice, D. W., Rob, A., & Stillman, T. J. (1992) *J. Mol. Biol. 228*, 662−671.
Becker, A. B., & Roth, R. A. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 3835−3839.
Berchtold, H., Reshetnikova, L., Reiser, C., Schirmer, N., Sprinzl, M., & Hilgenfeld, R. (1993) *Nature (London) 365*, 126−132.
Bourne, H. R., Sanders, D. A., & McCormick, F. (1991) *Nature (London) 349*, 117−127.
Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.
Burns, R. G. (1991) *Cell Motil. Cytoskeleton 20*, 181−189.
Burns, R. G., Farrell, K. W., & Surridge, C. D. (1993) *Ciba Found. Symp. 176*, 248−267.
Chavan, A., Kim, H., Haley, B., & Watt, D. (1990) *Bioconjugate Chem. 1*, 337−344.
Chiang, H. L., & Dice, J. F. (1988) *J. Biol. Chem. 263*, 6797−6805.
Clanton, D. J., Hattori, S., & Shih, T. Y. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 5076−5080.
Correia, J. J., Baty, L. T., & Williams, R. C. J. (1987) *J. Biol. Chem. 262*, 17278−84.
Davis, A., Sage, C. R., Wilson, L., & Farrell, K. W. (1993) *Biochemistry 32*, 8823−8835.
Davis, A., Sage, C. R., Dougherty, C. A., & Farrell, K. W. (1994) *Science 264*, 839−842.
de Boer, P., Crossley, R., & Rothfield, L. (1992) *Nature (London) 359*, 254−256.
Der, C. J., Pan, B. T., & Cooper, G. M. (1986) *Mol. Cell. Biol. 6*, 3291−3294.
Dreusicke, D., & Schulz, G. E. (1986) *FEBS Lett. 208*, 301−304.

Dustin, P. (1984) *Microtubules*, p 452, Springer-Verlag, New York.
Farr, G. W., & Sternlicht, H. (1992) *J. Mol. Biol. 227*, 307−321.
Feig, L. A., Pan, B. T., Roberts, T. M., & Cooper, G. M. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 4607−4611.
Fitzgerald-Hayes, M., Clarke, L., & Carbon, J. (1982) *Cell 29*, 235−244.
Flaherty, K. M. McKay, D. B., Kabsch, W., & Holmes, K. C. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 5041−5045.
Garces, E., & Cleland, W. W. (1969) *Biochemistry 8*, 633−640.
Geahlen, R. L., & Haley, B. E. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4375−4377.
Gilman, A. G. (1987) *Annu. Rev. Biochem. 56*, 615−649.
Hesse, J., Maruta, H., & Isenberg, G. (1985) *FEBS Lett. 179*, 91−95.
Hesse, J., Thierauf, M., & Postingl, H. (1987) *J. Biol. Chem. 262*, 15472−15475.
Hummel, J. P., & Dreyer, W. J. (1962) *Biochim. Biophys. Acta 63*, 530−532.
Hwang, Y.-W.; & Miller, D. L. (1987) *J. Biol. Chem. 262*, 13081−13085.
Hwang, Y., Jurnak, F., & Miller, D. (1989) in *The guanine nucleotide binding proteins: common structure and functional properties* (Bosch, L., Kraal, B., & Parmeggiani, A., Eds.) pp 77−85, Plenum, New York.
Ito, H., Fukuda, Y., Murata, K., & Kimura, A. (1983). *J. Bacteriol. 153*, 163−168.
Jacobs, M. (1975) *Ann. N.Y. Acad. Sci. 253*, 562−572.
Jayaram, B., & Haley, B. E. (1994) *J. Biol. Chem. 269*, 3233−3242.
John, J., Rensland, H., Schlichting, I., Vetter, I., Borasio, G. D., Goody, R. S., & Wittinghofer, A. (1993) *J. Biol. Chem. 268*, 923−929.
Jurnak, F. (1985) *Science 230*, 32−36.
Kassir, Y., & Simchen, G. (1991) *Methods Enzymol. 194*, 94−110.
Katz, W., & Solomon, F. (1988) *Mol. Cell. Biol. 8*, 2730−2736.
Kessler, F., Blobel, G., Patel, H. A., Schnell, D. J. (1994) *Science 266*, 1035−1039.
Kim, H., Ponstingl, H., & Hayley, B. E. (1987) *Fed. Prod. 46*, 22−29.
Kirschner, M., & Mitchison, T. (1986) *Cell 45*, 329−342.
Kunkel, T. A., Roberts, J. D., & Zakour, R. A. (1987) *Methods Enzymol. 154*, 367−382.
Levi, A., Cimino, M., Mercanti, D., & Calissano, P. (1974) *Biochim. Biophys. Acta 365*, 450−453.
Linse, K., & Mandelkow, E.-M. (1988) *J. Biol. Chem. 263*, 15205−15210.
Little, M., & Seehaus, T. (1988) *Comp. Biochem. Physiol. 90*, 655−670.
Low, A., Sprinz, M., & Faulhammer, H. G. (1993) *Eur. J. Biochem. 215*, 473−479.
Luduena, R. F., Shooter, E. M., & Wilson, L. (1977) *J. Biol. Chem. 252*, 7006−7014.
Maccioni, R. B., & Seeds, N. W. (1983) *Biochemistry 22*, 1572−1579.
Mandelkow, E-M., Herrmann, M., & Ruhl, U. (1985) *J. Mol. Biol. 185*, 311−327.
Mejillano, M. R., & Himes, R. H. (1991) *Arch. Biochem. Biophys. 291*, 356−362.
Mitchison, T. J., & Kirschner, M. (1984) *Nature (London) 312*, 237−242.
Mukherjee, A., & Lutkenhaus, J. (1994) *J. Bacteriol. 176*, 2754−2756.
Nakaoka, H., Perez, D. M., Jin Baek, K., Das, T., Husain, A., Misono, K., Im, M.-J., & Graham, R. M. (1994) *Science 264*, 1593−1596.
Nath, J. P., Eagle, G. R., & Himes, R. H. (1985) *Biochemistry 24*, 1555−1560.
Noel, J. P., Hamm, H. E., & Sigler, P. B. (1993) *Nature (London) 366*, 654−663.
Pai, E. F., Krengel, U., Petsko, G. A., Goody, R. S., Kabsch, W., & Wittinghofer, A. (1990) *EMBO J. 9*, 2351−2359.
Pearl, L. (1993) *Nature (London) 362*, 24.
Peter, M. E., Wittmann-Liebold, B., & Sprinzl, M. (1988) *Biochemistyr 27*, 9132−9139.
RayChaudhuri, D., & Park, J. T. (1992) *Nature (London) 359*, 251−254.
Reijo, R. A., Cooper, E. M., Beagle, G. J., & Huffaker, T. C. (1994) *Mol. Biol. Cell 5*, 29−43.
Sage, C., Davis, A., Dougherty, C., Sullivan, K., & Farrell, K. (1995) *Cell Motil. Cytoskeleton* (in press).
Schlichting, I., Almo, S. C., Rapp, G., Wilson, K., Petratos, K., Lentfer, A., Wittinghofer, A., Kabsch, W., Pai, E. F., Petsko, G. A. & Goody, R. S. (1990) *Nature (London) 345*, 309−315.
Shivanna, B. D., Mejillano, M. R., Williams, T. D., & Himes, R. H. (1993) *J. Biol. Chem. 268*, 127−132.
Sigal, I. S., Gibbs, J. B., D'Alonzo, J. S., Temeles, G. L., Wolanski, B. S., Socher, S. H., & Scolnick, E. M. (1986) *Prob. Natl. Acad. Sci. U.S.A. 83*, 952−956.
Steiner, M. (1984) *Biochem. Biophys. Res. Commun. 123*, 92−99.
Sternlicht, H., Yaffe, M. B., & Farr, G. W. (1987) *FEBS Lett. 214*, 226−235.
Takeuchi, Y., Birckbichler, P. J., Patterson, M. K. J., & Lee, K. N. (1992) *FEBS Lett. 307*, 177−180.
Valencia, A., Chardin, P., Wittinghofer, A., & Sander, C. (1991) *Biochemistry 30*, 4637−4648.
Weijland, A., & Parmeggiani, A. (1993) *Science 259*, 1311−1314.
Zweig, G., & Whitaker, J. R. (1971) in *Paper chromatography and electrophoresis*, pp 248−282, Academic Press, New York.

BI950304B