Molecular Biology of the Cell
Vol. 10, 2987–3001, September 1999

Functional Dissection and Hierarchy of Tubulin-folding Cofactor Homologues in Fission Yeast

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Submitted May 7, 1999; Accepted July 1, 1999
Monitoring Editor: Tim Stearns

We describe the isolation of fission yeast homologues of tubulin-folding cofactors B (Alp11) and E (Alp21), which are essential for cell viability and the maintenance of microtubules. $Alp11^B$ contains the glycine-rich motif (the CLIP-170 domain) involved in microtubular functions, whereas, unlike mammalian cofactor E, $Alp21^E$ does not. Both mammalian and yeast cofactor E, however, do contain leucine-rich repeats. Immunoprecipitation analysis shows that $Alp11^B$ interacts with both α-tubulin and $Alp21^E$, but not with the cofactor D homologue Alp1, whereas $Alp21^E$ also interacts with $Alp1^D$. The cellular amount of α-tubulin is decreased in both *alp1* and *alp11* mutants. Overproduction of $Alp11^B$ results in cell lethality and the disappearance of microtubules, which is rescued by co-overproduction of α-tubulin. Both full-length $Alp11^B$ and the C-terminal third containing the CLIP-170 domain localize in the cytoplasm, and this domain is required for efficient binding to α-tubulin. Deletion of *alp11* is suppressed by multicopy plasmids containing either $alp21^+$ or $alp1^+$, whereas *alp21* deletion is rescued by overexpression of $alp1^+$ but not $alp11^+$. Finally, the *alp1* mutant is not complemented by either $alp11^+$ or $alp21^+$. The results suggest that cofactors operate in a linear pathway ($Alp11^B$-$Alp21^E$-$Alp1^D$), each with distinct roles.

INTRODUCTION

Microtubules (MTs) are ubiquitous structures important for a wide variety of cellular processes, including motility, chromosome separation, protein and mRNA transport, and cell morphogenesis. They are composed of heterodimers of α- and β-tubulins, which are evolutionarily highly conserved. MTs undergo morphological alterations in cell cycle– and differentiation stage-specific manners; elucidation of the underlying molecular mechanisms is crucial to understanding how MTs regulate diverse cellular processes (Mitchison and Kirschner, 1986; Hyman and Karsenti, 1996). MTs are known to be regulated at several distinct levels in the cell. These include posttranscriptional autoregulation (Bachurski *et al.*, 1994; Gonzalez-Garay and Cabral, 1996), treadmilling (Waterman-Storer and Salmon, 1997), assembly and disassembly through dynamic instability or severing reactions (Mitchison and Kirschner, 1984; Horio and Hotani, 1986; McNally and Vale, 1993; Belmont and Mitchison, 1996), and stabilization through a group of microtubule-associated proteins (MAPs) (Mandelkow and Mandelkow, 1995). MAPs are further classified into several categories depending on amino acid sequence homology and their biochemical characteristics. For example, classic MAPs consist of tau and MAP1, MAP2, and MAP4. MAPs in a broad sense include motor proteins such as kinesin and dynein (Hirokawa, 1998). Also, several proteins, including CLIP-170/restin and the $p150^{Glued}$ component of the dynactin complex, share a common motif of ~50 amino acid residues, and some of these members have been shown to bind MTs (Gill *et al.*, 1991; Bilbe *et al.*, 1992; Pierre *et al.*, 1992; Riehemann and Sorg, 1993).

In addition to these controls, the biogenesis of MTs is regulated by molecular chaperones, members of the GroEL- and Hsp60-related chaperonins (CCT/TriC/c-cpn) (Rommelaere *et al.*, 1993; Kubota *et al.*, 1994). Of particular interest is that, although newly synthesized actin and γ-tubulin are folded properly in vitro mainly by CCT/TriC/c-cpn (Gao *et al.*, 1992; Melki *et al.*, 1993; Geissler *et al.*, 1998; Vainberg *et al.*, 1998), folding reactions for α- and β-tubulin molecules are of far greater complexity. In addition to CCT/TriC/c-cpn, a group of proteins called cofactors are required for the production of assembly-competent α/β-tubulin heterodimers (Gao *et al.*, 1993, 1994). Molecular cloning of these cofactors from vertebrates has revealed that cofactors comprise multiple proteins, cofactors A, B, C, D, and E (Llosa *et al.*, 1996; Melki *et al.*, 1996; Tian *et al.*, 1996, 1997). Interestingly, both cofactors B and E contain the conserved motif that is found

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Abbreviations used: GFP, green fluorescent protein; HA, hemagglutinin; MT, microtubule.

in several proteins, including CLIP-170 and the dynactin subunit mentioned above (Tian *et al.*, 1996, 1997).

According to biochemical analysis performed in mammalian systems with tubulins expressed in vitro with purified chaperonins and cofactors, the pathways leading to correctly folded α/β-tubulin heterodimers are as follows. Newly translated free tubulins first enter a 900-kDa toroidal complex consisting of several chaperonin subunits (Lewis *et al.*, 1992; Yaffe *et al.*, 1992). After release from the chaperonin complex, α- and β-tubulins are captured by cofactors B and A, respectively, which subsequently are replaced by cofactors E and D. Then, the two pathways (α-tubulin-cofactor E and β-tubulin-cofactor D) converge, forming a complex consisting of all four molecules. Finally, cofactor C joins and, upon GTP hydrolysis, assembly-competent α/β-tubulin heterodimers are released. Cofactors D and E are indispensable and play parallel roles in the folding reactions of β- and α-tubulin, respectively (Lewis *et al.*, 1997).

Cofactors are also evolutionarily conserved from humans to yeasts. In budding yeast, homologues of all the cofactors except cofactor C are found in the genome, and mutants and corresponding genes were independently isolated in genetic screens to identify genes involved in MT function (Hoyt *et al.*, 1990, 1997; Stearns *et al.*, 1990; Ursic and Culbertson, 1991; Archer *et al.*, 1995; Tian *et al.*, 1997). These analyses have shown that, surprisingly, none of the cofactor homologues are essential for cell viability in budding yeast; MTs appear to be capable of assembling in the absence of these cofactors, although modest defects in MT function are observed in mutants defective in these homologues, such as hypersensitivity to MT-destabilizing drugs and cold-sensitive growth. This raises the question as to the function of these cofactors in MT biogenesis: do cofactors play a crucial role in MT function in vivo? Analysis in fission yeast, however, has given a quite different view. The cofactor D homologue–encoding *alp1*$^+$ gene is essential, and temperature-sensitive *alp1* mutants show a variety of phenotypes attributed to defects in MT function, such as abnormal bent or branched morphology, displacement of the nucleus and septum, and mitotic delay. More importantly, MT structures become highly fragile and no intact MTs are observed at the restrictive temperature, demonstrating that cofactors are required for MT function in fission yeast (Hirata *et al.*, 1998).

alp1 was originally identified as one of 15 loci (*alp1* to *alp15*), temperature-sensitive mutations of which result in growth polarity defects, including curved, bent, or branched cell shape at the restrictive temperature. Previous analysis has shown that four loci (*alp1*$^+$, *alp2*$^+$, *alp11*$^+$, and *alp12*$^+$) are responsible for the maintenance of MT structures (Radcliffe *et al.*, 1998). As described above, *alp1*$^+$ encodes a homologue of cofactor D and *alp2*$^+$ and *alp12*$^+$ are allelic to *atb2*$^+$ and *nda3*$^+$, encoding α2-tubulin and β-tubulin, respectively.

In the current study, we have isolated and characterized the fourth gene, *alp11*$^+$, which turns out to encode a homologue of cofactor B. Furthermore, we have isolated a homologue of cofactor E (*alp21*$^+$) as a multicopy suppressor of the temperature-sensitive *alp11* mutant. Genetic analysis indicates that, consistent with findings for *alp1*$^+$, both *alp11*$^+$ and *alp21*$^+$ are essential for MT function and cell viability. In agreement with the result obtained from mammalian in vitro systems, we show that Alp21^E functions downstream of Alp11^B. However, contrary to the notion that cofactors D and E act in a parallel manner, our analysis suggests that in fission yeast Alp1^D acts downstream of Alp21^E. Structure-function relationships of these cofactors are discussed in terms of evolutionary conservation in the biogenesis and regulation of MTs.

Table 1. Strain list

Strain	Genotype	Derivation
HM123	*h−leu1*	Our stock
DH2-8D	*h*$^-$*leu1alp1-1315*	Hirata *et al.* (1998)
DH924	*h*$^-$*leu1alp11-924*	This study
ME1	*h*$^-$*/h*$^+$*leu1/leu1ura4/ura4his7/his7 ade6-210/ade6-216alp1::ura4*$^+$*/+*	Hirata *et al.* (1998)
PR6	*h−leu1ura4alp11-924*	This study
PR7	*h*$^-$*/h*$^+$*leu1/leu1ura4/ura4his7/his7 ade6-M210/ade6-M216alp11::ura4*$^+$*/+*	This study
PR8	*h*$^-$*/h*$^+$*leu1/leu1ura4/ura4his7/his7 ade6-M210/ade6-M216alp21::ura4*$^+$*/+*	This study
PR9[a]	*h*$^-$*leu1ura4his7ade6alp11::ura4*$^+$	This study
PR10	*h*$^-$*leu1ura4his7ade6alp21::ura4*$^+$ containing pAL100 (*alp1*$^+$)	This study
PR11	*h*$^-$*leu1alp11*$^+$*-GFP-ura4*$^+$	This study
PR12	*h*$^-$*leu1alp21*$^+$*-GFP-ura4*$^+$	This study
PR13	*h*$^-$*/h*$^+$*leu1/leu1ura4/ura4his7/his7 ade6-M210/ade6-M216alp11*$_{1-163}$*::ura4*$^+$*/+*	This study
PR14	*h*$^-$*leu1ura4his7ade6alp11*$_{1-163}$*::ura4*$^+$	This study
PR15	*h*$^-$*leu1ura4his7ade6alp1*$^+$*-HA-kan*	This study
PR16	*h*$^-$*leu1ura4his7ade6alp21*$^+$*-GFP-ura4*$^+$*alp1*$^+$*-HA-kan*	This study
PR17	*h*$^-$*leu1ura4his7ade6alp11*$_{1-163}$*::ura4*$^+$*alp21*$^+$*-GFP-ura4+*	This study
PR18	*h*$^-$*leu1alp21*$^+$*-GFP-ura4*$^+$*pop3*$^+$*-HA-kan*	This study

[a] This strain was spontaneously obtained from tetrads derived from PR7, which could be propagated, possibly ascribable to the occurrence of some suppressor mutation.

MATERIALS AND METHODS

Strains, Media, and Genetic Methods

The strains used in this study are listed in Table 1. YPD (2% dextrose, 2% polypeptone, and 1% yeast extract) and YE5S (Moreno *et al.*, 1991) were used as rich media. The standard methods were followed as described (Moreno *et al.*, 1991).

Cloning of the alp11$^+$ *and alp21*$^+$ *Genes*

A *Schizosaccharomyces pombe* genomic library was used for the isolation of genes that complemented the temperature-sensitive *alp11-924* mutant (PR6). Two of 20,000 colonies were capable of growing at 36°C. Plasmid DNAs were recovered from *Escherichia coli*, and restriction mapping indicated that two different plasmids (pCR1 and pCR11; the corresponding genes were designated *alp11*$^+$ and *alp21*$^+$, respectively) were isolated. Allelism between a gene on pCR1 and *alp11*$^+$ was determined by tetrad analysis between the viable Δ*alp11* disruptant (PR9; see below) and the *alp11-924* strain (PR6). Ts$^+$ (wild-type) recombinants did not appear among 13 tetrads.

Table 2. Oligonucleotides used in this study

Name	Sequence (5′–3′)
PO1	TCGACATATGAATGAGATAACTCT
PO2	TCGAAGATCTTCAAAGACCTTCTAAAA
PO3	TCGACATATGCATATATCCACTGGA
PO4	TCGAGGATCCTTAGACGTTTCCACTTT
PO5	TATGGGATCCTTACTCTCCTGCTGCACAGC
PO6	TCGACATATGGACTTTGAAGCAAGTAAAG
PO7	TATGCTGCAGTAATCATATATCTTAAG
PO8	TATGAGATCTATTAACATGGAACCTTG
PO9	TCGACATATGATTAACAATTTGAA
PO10	TATGCATATGAGAGAGATCATTTCC
PO11	TATGGGATCCTTAGTACTCTTCTTC

Nucleic Acids Preparation and Manipulation

Standard molecular biology techniques were followed as described (Sambrook *et al.*, 1989). Enzymes were used as recommended by the suppliers (New England Biolabs, Beverly, MA). Nucleotide sequence data reported in this paper are in the DDBJ/EMBL/GenBank databases under accession numbers AB008750 (*alp11*$^+$) and AB008749 (*alp21*$^+$).

Gene Disruption

The *alp11*$^+$ and *alp21*$^+$ genes were deleted with the use of PCR-generated fragments (Bähler *et al.*, 1998). Dissection of asci from heterozygous diploid cells (PR7 and PR8, respectively) showed that both genes were essential for cell viability, as two viable and two nonviable spores were obtained from 20 tetrads dissected and viable colonies were Ura$^-$ in both cases. Extended incubation (5 d at 29°C) of tetrads derived from PR7 led to the appearance of tiny colonies (Ura$^+$) at a frequency of around 50%.

Chromosomal Deletion of alp11 CLIP-170 Sequence

Oligonucleotides (100 bases long) were designed to create an *alp11*-truncation mutant with the use of a PCR-generated fragment (Bähler *et al.*, 1998) in which a stop codon was introduced at amino acid 164 in one of the chromosomal *alp11*$^+$ genes in a diploid (PR13; Table 1).

Overexpression and Epitope Tagging

The entire ORFs of the *alp11*$^+$ and *alp21*$^+$ genes were cloned by PCR into pREP1 under control of the *nmt1* promoter (Maundrell, 1990): oligonucleotides PO1 and PO2 (Table 2) were used for *alp11*$^+$, yielding pREP-*alp11*$^+$, and PO3 and PO4 were used for *alp21*$^+$, yielding pREP-*alp21*$^+$. Both were functional, as they complemented the temperature-sensitive *alp11* mutant. Truncated *alp11* genes carried on pREP1 encoding amino acids 1–163 or 131–234 were made by PCR with the use of the following oligonucleotides: PO1 and PO5 or PO2 and PO6 (Table 2), designated pREP-alp11$_{1–163}$ and pREP-alp11$_{131–234}$, respectively.

C-terminal tagging of the chromosomal *alp11*$^+$, *alp21*$^+$, and *alp1*$^+$ genes with *GFP* and the hemagglutinin (HA) peptide was performed with the use of a PCR-generated fragment (PR11, PR12, and PR15; Table 1) (Bähler *et al.*, 1998). The entire ORF of *alp11*$^+$ was also subcloned into pREP42GFP (Craven *et al.*, 1998), yielding pREP42-GFP-*alp11*$^+$. The tagged gene was functional, as pREP42-GFP-*alp11*$^+$ rescued the temperature-sensitive *alp11-924* mutant. A truncated *alp11* gene, which encodes the CLIP-170 domain, was inserted into pREP42GFP, yielding pREP42-GFP-alp11$_{131–124}$. The entire ORF of *atb2*$^+$ was also subcloned into pREP2 with the use of oligonucleotides PO10 and PO11 (Table 2), yielding pREP2-*atb2*$^+$. pREP2-*nda3*$^+$ was described previously (Hirata *et al.*, 1998).

Immunochemical Assays

Rabbit polyclonal anti-Alp11 and anti-Alp21 antibodies were prepared as follows. To express Alp11 protein, a 700-base pair fragment (corresponding to the entire ORF; PO1 and PO2 were used for PCR) was inserted into pET10c (Invitrogen, Carlsbad, CA). In the case of Alp21, a 740-base pair fragment (corresponding to amino acid residues 269–512; PO4 and PO9 were used for PCR) (Table 2) was used. Immunoblotting was performed with crude sera or affinity-purified antibodies. Affinity-purified rabbit polyclonal anti-Alp1 antibody (Hirata *et al.*, 1998), mouse monoclonal anti-α-tubulin antibody (TAT-1; provided by Dr. Keith Gull), mouse monoclonal anti-β-tubulin antibody (KMX-1; provided by Dr. Keith Gull), mouse monoclonal anti-Cdc2 antibody (provided by Dr. Hiroyuki Yamano), mouse monoclonal anti-GFP antibody (used for immunoblotting; 8362-1, Clontech, Palo Alto, CA), and rabbit polyclonal anti-GFP antibody (used for immunoprecipitation; provided by Dr. Kenneth Sawin) were also used. Horseradish peroxidase–conjugated goat anti-rabbit immunoglobulin G, goat anti-mouse immunoglobulin G (Bio-Rad Laboratories, Richmond, CA), and a chemiluminescence system (ECL, Amersham, Arlington Heights, IL) were used to detect bound antibody. Fission yeast whole cell extracts were prepared with glass beads to disrupt cells as described by Kominami *et al.* (1998). For immunoprecipitation, PME buffer (0.1 M piperazine-*N*,*N*′-bis[2-ethanesulfonic acid], 2 mM EGTA, 1 mM $MgCl_2$, pH 6.9), plus a cocktail of inhibitors as described (Vega *et al.*, 1998), was used.

Gel Filtration Chromatography

Gel filtration chromatography was performed on a Superose-6 column by fast-performance liquid chromatography (Pharmacia Biotech, Piscataway, NJ) in buffer A (20 mM Tris-HCl, pH 7.5, 20% glycerol, 0.1 mM EDTA, 1 mM mercaptoethanol, 5 mM ATP, plus a cocktail of inhibitors). The column was equilibrated with 2 column volumes of buffer A containing 100 mM NaCl. To determine molecular mass, a parallel column was run with standards consisting of dextran (2000 kDa), thyroglobulin (669 kDa), and α-amylase (232 kDa). Fractions (50 μl each) were separated by SDS-PAGE on 10% gels, and fractionated proteins were detected with individual antibodies.

Indirect Immunofluorescence Microscopy

Cells were fixed with methanol, and primary antibodies (anti-Alp11, TAT-1, or anti-GFP) were applied, followed by Cy3-conjugated goat anti-rabbit immunoglobulin G (Sigma) or fluorescein-linked sheep anti-mouse immunoglobulin G (Amersham).

Cross-Suppression Experiments

Diploids heterozygous for *alp1*$^+$ (ME1), *alp11*$^+$ (PR7), or *alp21*$^+$ (PR8) were transformed with multicopy plasmids containing *alp1*$^+$ (pAL100 and pREP-*alp1*$^+$; Hirata *et al.*, 1998), *alp11*$^+$ (pREP-*alp11*$^+$), or *alp21*$^+$ (pREP-*alp21*$^+$). Leu$^+$ transformants were allowed to sporulate, and free spores were directly plated on minimal plates supplemented with adenine and histidine with or without uracil and thiamine. If Ura$^+$Leu$^+$ haploid colonies were obtained in which plasmids (Leu$^+$ prototrophy) were mitotically stable, this plasmid was assigned to be capable of suppressing deletion of the gene of interest. Δ*alp11* (PR9) and temperature-sensitive *alp1* mutants (DH2-8D) were also used.

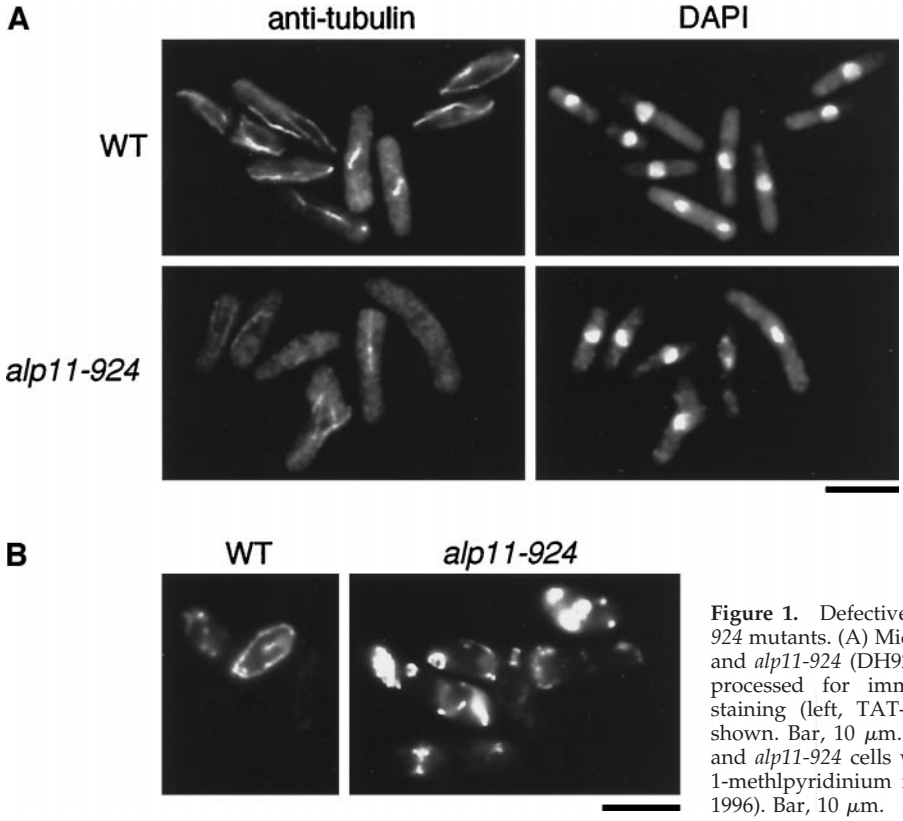

Figure 1. Defective phenotypes of temperature-sensitive *alp11-924* mutants. (A) Microtubule structures. Wild-type (WT; HM123) and *alp11-924* (DH924) cells were incubated at 36°C for 6 h and processed for immunofluorescence microscopy. Anti-tubulin staining (left, TAT-1) and nuclear staining (right, DAPI) are shown. Bar, 10 μm. (B) Distribution of mitochondria. Wild-type and *alp11-924* cells were stained with 2-(4-dimethyaminostyryl)-1-methlpyridinium iodide, as described previously (Yaffe *et al.*, 1996). Bar, 10 μm.

RESULTS

The alp11-924 Mutation Results in Loss of Microtubule Structures and Growth Polarity Defects

The *alp11-924* mutant was isolated in a screen for genes involved in growth polarity control (Hirata *et al.*, 1998). Mutant cells divided once at the restrictive temperature and showed bent or branched morphology (26 and 5%, respectively, after 8 h at 36°C). Wild-type cells do not show this type of abnormal morphology. Mitotic delay was also evident, as condensed chromosomes were observed to a high extent (30%, in contrast to the only 1–2% of exponentially growing wild-type cells that show condensed chromosomes). The percentage of septated cells increased from 20% (at 26°C) to 31% (6 h at 36°C), of which 62% had septa displaced from the center of the cell. The position of the nucleus was also often (24%) displaced from the medial region, and 3% of cells were anucleate, attributed to asymmetrical cell division.

These phenotypes are very similar to those of temperature-sensitive *alp1*, *alp2/atb2*, and *alp12/nda3* mutants, which are defective in MT function (Hirata *et al.*, 1998; Radcliffe *et al.*, 1998). To examine MT structures in the temperature-sensitive *alp11-924* mutant, MTs were stained with anti-α-tubulin antibody. As shown in Figure 1A, MTs became fragile in this mutant. No intact MTs were observed; instead, either none, short, or misoriented MTs clustered around the nucleus remained. Also, mitochondria, the localization of which is dependent on and often coincident with cytoplasmic MTs in fission yeast (Yaffe *et al.*, 1996), became aggregated just as in tubulin mutants (Figure 1B). In line with the defects in MTs, *alp11-924* cells were hypersensitive to MT-depolymerizing drugs such as thiabendazole, even at the permissive temperature (26°C; our unpublished results). These results showed that *alp11*$^+$ is required for the maintenance or establishment of MTs.

alp11$^+$ *and a Multicopy Suppressor alp21*$^+$ *Encode Homologues of Tubulin-folding Cofactors B and E, Respectively*

A fission yeast genomic library was used to isolate genes that complemented the temperature-sensitive *alp11-924* mutation. Two different plasmids (pCR1 and pCR11) that contained nonoverlapping inserts were isolated. Genetic linkage analysis (see MATERIALS AND METHODS) indicated that the gene in pCR1 is *alp11*$^+$ and the gene in pCR11 is a multicopy suppressor (designated *alp21*$^+$). Nucleotide sequencing showed that *alp11*$^+$ and *alp21*$^+$ encode fission yeast homologues of cofactors B and E, respectively (Tian *et al.*, 1996, 1997). Amino acid sequence identity is 35% between human cofactor B and Alp11 and 27% between cofactor E and Alp21.

Comparison of the amino acid sequences of fission yeast and vertebrate cofactors B and E illuminated some interesting features of structural conservation and divergence through evolution. As in cofactor B from other organisms, Alp11^B contains a glycine-rich region of around 50 amino acid residues that shows a high degree of homology to a motif found in a group of proteins related to MT-dependent

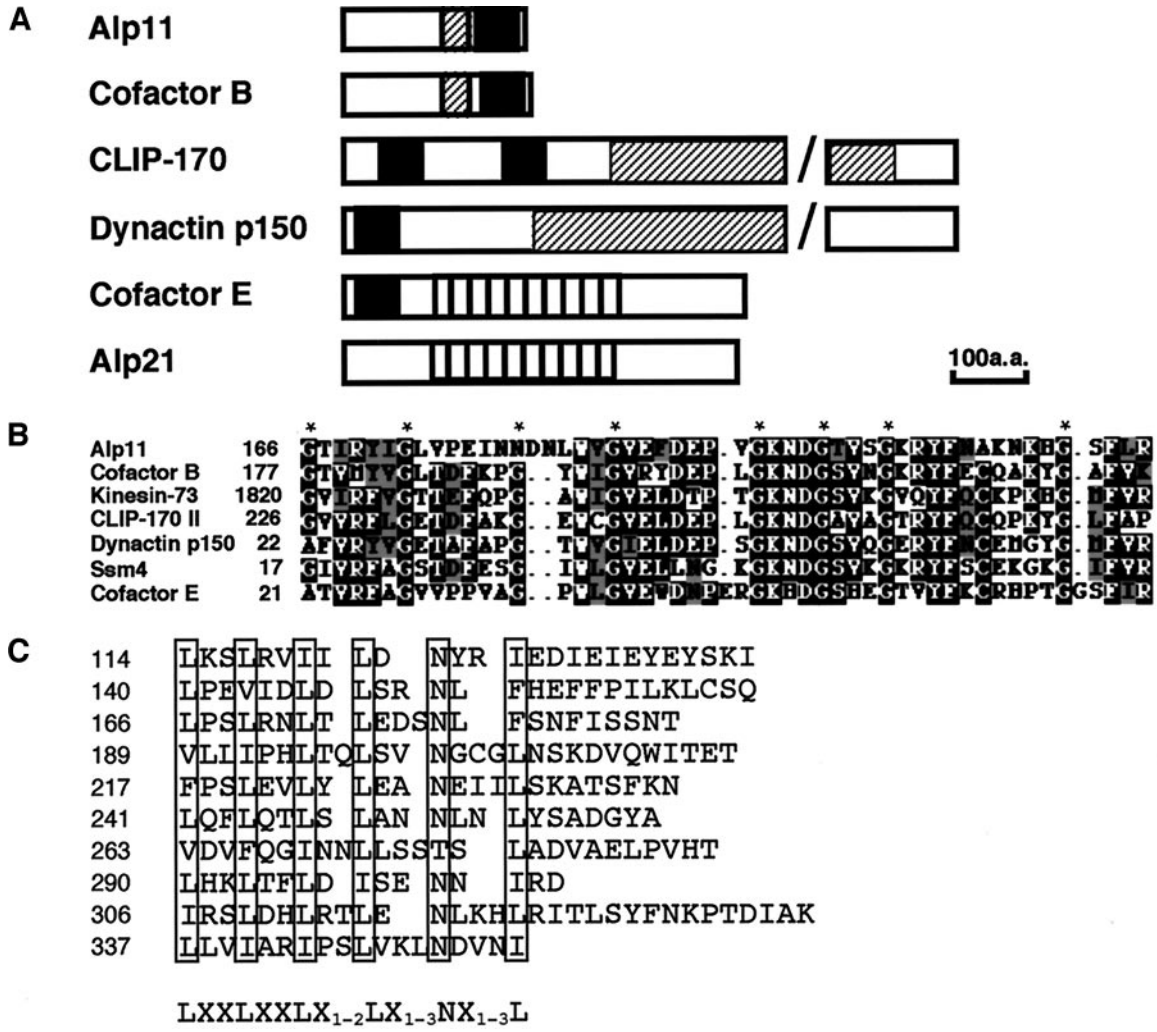

Figure 2. Structural motifs found in cofactors B and E. (A) Overall structural organization of various proteins containing the glycine-rich tubulin-interacting (CLIP-170) domain and/or leucine-rich repeats (LRRs). The CLIP-170 domain and LRR units are shown in closed and small open boxes, respectively. Coiled-coil regions are shown with hatched boxes. (B) Sequence alignment of the CLIP-170 domain. Invariant glycines are indicated with asterisks. (C) Sequence alignment of LRRs in Alp21. Consensus amino acid residues are boxed, and a repeating unit is shown below.

processes. These include CLIP-170/restin, dynactin, kinesin-73, vertebrate cofactor E, and fission yeast Ssm4 (Figure 2, A and B; hereafter this domain will be called the CLIP-170 domain) (Gill *et al.*, 1991; Bilbe *et al.*, 1992; Pierre *et al.*, 1992; Tian *et al.*, 1996; Li *et al.*, 1997; Yamashita *et al.*, 1997). Unlike vertebrate cofactor E and also the budding yeast homologue Pac2 (Hoyt *et al.*, 1997), Alp21^{E} does not contain the analogous domain in the N-terminal region (Figure 2A). However, we did find that both vertebrate cofactor E and the yeast homologues contain leucine-rich repeating (LRR) motifs in their central regions. The repeating motif consists of the consensus $LX_2LX_2LX_{1-2}LX_{1-3}NX_{1-3}L$ (where X is any amino acid), and 10 repeats are found in Alp21 and vertebrate cofactor E (Figure 2, A and C). LRRs are believed to mediate protein-protein interactions (Kobe and Deisenhofer, 1994).

Gene Disruption of the alp11$^+$ and alp21$^+$ Genes

Gene disruption experiments showed that both genes were essential for cell viability (Figure 3, A and B, left). Microscopic observation of inviable spores indicated that in both cases spores were capable of germinating; they divided one or two times and arrested, with abnormally bent or branched cell morphology (Figure 3B, right). We also noticed that after longer incubation (5 d at 29°C) ~50% of *alp11*-deleted spores formed tiny Ura$^+$ (Δ*alp11*) colonies (Figure 3A, left, arrow), some of which managed to survive upon subculture. Cells from these colonies were defective in cell morphology (Figure 3A, right) and showed temperature-sensitive and also cold-sensitive growth. We have not genetically analyzed these viable strains further but suspect that a suppressor mutation(s) may have arisen that rescues

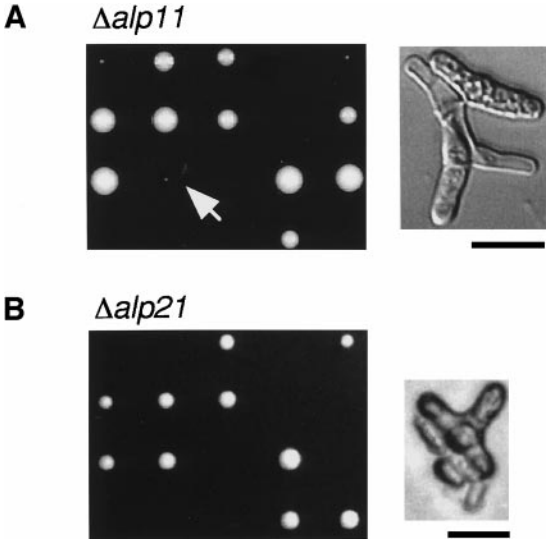

Figure 3. Gene disruption of *alp11*$^+$ and *alp21*$^+$ genes. Tetrad analysis of diploids heterozygous for *alp11* (A; PR7) or *alp21* (B; PR8) is shown. Note that tiny colonies grow from some tetrads of PR7 (arrow). Cell morphology of an *alp11*-deleted haploid cell (A) and an *alp21*-deleted cell (B) is shown. Bars, 10 μm.

Δ*alp11*. This result showed that, like cofactor D–encoding *alp1*$^+$ (Hirata *et al.*, 1998), cofactors B– and E–encoding *alp11*$^+$ and *alp21*$^+$ genes are essential for cell viability.

Physical Interaction among Cofactors and α-Tubulin

Polyclonal antibodies against Alp11^B and Alp21^E were produced. Immunoblotting with the use of anti-Alp11 antibody against total cell extracts prepared from wild type identified a single band of 35 kDa (p35^{alp11}; Figure 4A, lane 1), which was missing in Δ*alp11* and highly enhanced in *alp11*$^+$-overexpressing cells (Figure 4A, lanes 2 and 3). In the case of anti-Alp21 antibody, sera were not specific enough to identify the *alp21*$^+$ gene product derived from a single chromosomal gene. Therefore, the chromosomal *alp21*$^+$ gene (and also *alp11*$^+$) was tagged with *GFP* at the C terminus. Also the HA epitope was used to tag the chromosomal *alp1*$^+$ gene (see MATERIALS AND METHODS). Tagging did not interfere with Alp11^B, Alp21^E, or Alp1^D function, as haploid strains containing integrated *GFP* or *HA* behaved like wild-type cells. Immunoblotting with anti-GFP antibody showed a specific band of around 90 kDa in an *alp21*$^+$-GFP strain (Figure 4B, lane 1). This band was missing in the untagged and *alp11*$^+$-GFP strains (Figure 4B, lanes 2 and 3) and highly increased in a strain carrying *alp21*$^+$-GFP on a multicopy plasmid (Figure 4B, lane 4). Alp11^B-GFP was detected as a 60-kDa band (Figure 4B, lane 2). It should be noted that Alp11^B appears to exist in a greater amount than Alp21^E; the difference between the levels of Alp11^B-GFP and Alp21^E-GFP detected with the same anti-GFP antibody was at least 10-fold (Figure 4B; compare lanes 1 and 2).

To explore in vivo physical interactions between cofactors and tubulins, immunoprecipitation experiments were performed. Using the integrated *alp21*$^+$-*GFP* strain and anti-GFP antibody, we found that Alp21^E interacts with Alp1^D, Alp11^B, and α-tubulin (Figure 4C, lane 2). Reciprocal immunoprecipitation experiments with anti-Alp11 antibody confirmed the interaction between Alp11^B and Alp21^E and demonstrated that Alp11^B also coprecipitates with α-tubulin (Figure 4C, lanes 7 and 10). The interaction is specific, as no proteins were precipitated without the addition of antibody (Figure 4C, lanes 3 and 8) or when a Δ*alp11* (our unpublished results) or untagged strain was used (Figure 4C, lane 7). Alp11^B and α-tubulin are able to interact in the absence of Alp21^E, as these two proteins coimmunoprecipitated in a Δ*alp21* strain (Figure 4C, lane 9; kept viable by multicopy plasmids containing the *alp1*$^+$ gene [see below]). On the other hand, Alp11^B did not bind Alp1^D (Figure 4C, lane 7), nor did we see an interaction between Alp11^B/Alp21^E and β-tubulin (data not shown). The interaction described above was confirmed by independent immunoprecipitation experiments with the use of Alp1^D-HA–tagged strains. As shown in Figure 4D (lanes 5 and 7), Alp1^D-HA and Alp21^E-GFP, but not Alp11^B, coimmunoprecipitated. In summary, Alp11^B interacts with α-tubulin and Alp21^E but not Alp1^D, and Alp21^E also interacts with Alp1^D. These results are consistent with the notion that cofactor B specifically binds free α-tubulin monomers (Tian *et al.*, 1997; Feierbach *et al.*, 1999) and suggest that Alp21^E acts at an intermediate position in the α-tubulin–folding pathway between Alp11^B and Alp1^D.

Size Fractionation Analysis of Cofactors and Tubulins

We were interested in the native complex state of cofactors and tubulins in the cell. To this end, we analyzed the behavior of cofactors and tubulins by gel filtration chromatography. As shown in Figure 4E, it was found that Alp1^D and Alp21^E exist predominantly in two fractions, one larger (fractions 4–6, ~2000 kDa) and the other smaller (fractions 14–16, 400–500 kDa). The ratio of these two complexes appeared equal. In the case of Alp11^B and α-tubulin, most of these proteins exist in a complex(es) of a small size (fractions 17–22, 100–200 kDa), although the chromatographic patterns of these two proteins appeared not to be the same. In addition, a relatively minor population (<10%) of both Alp11^B and α-tubulin existed in a larger fraction that corresponded in size to Alp1^D and Alp21^E (fractions 4–6). This result suggests that Alp1^D and Alp21^E form stable dual complexes in the cell.

The Level of α-Tubulin Is Decreased in the alp1 and alp11 Mutants

The possibility that mutations in cofactors affected the level of free tubulin molecules was examined. It was found that the steady-state level of α-tubulin in temperature-sensitive *alp11-924* mutants was reduced at the restrictive temperature (Figure 5A, lanes 1 and 3) and that the level was lower than in the wild type even at the permissive temperature (Figure 5A, lane 2). The level of α-tubulin became comparable to that in wild-type cells when temperature-sensitive *alp11-924* mutants were transformed with plasmids containing *alp11*$^+$ (Figure 5, lane 4), whereas high-dose *alp21*$^+$ appeared not to restore the amount of α-tubulin (Figure 5, lane 5).

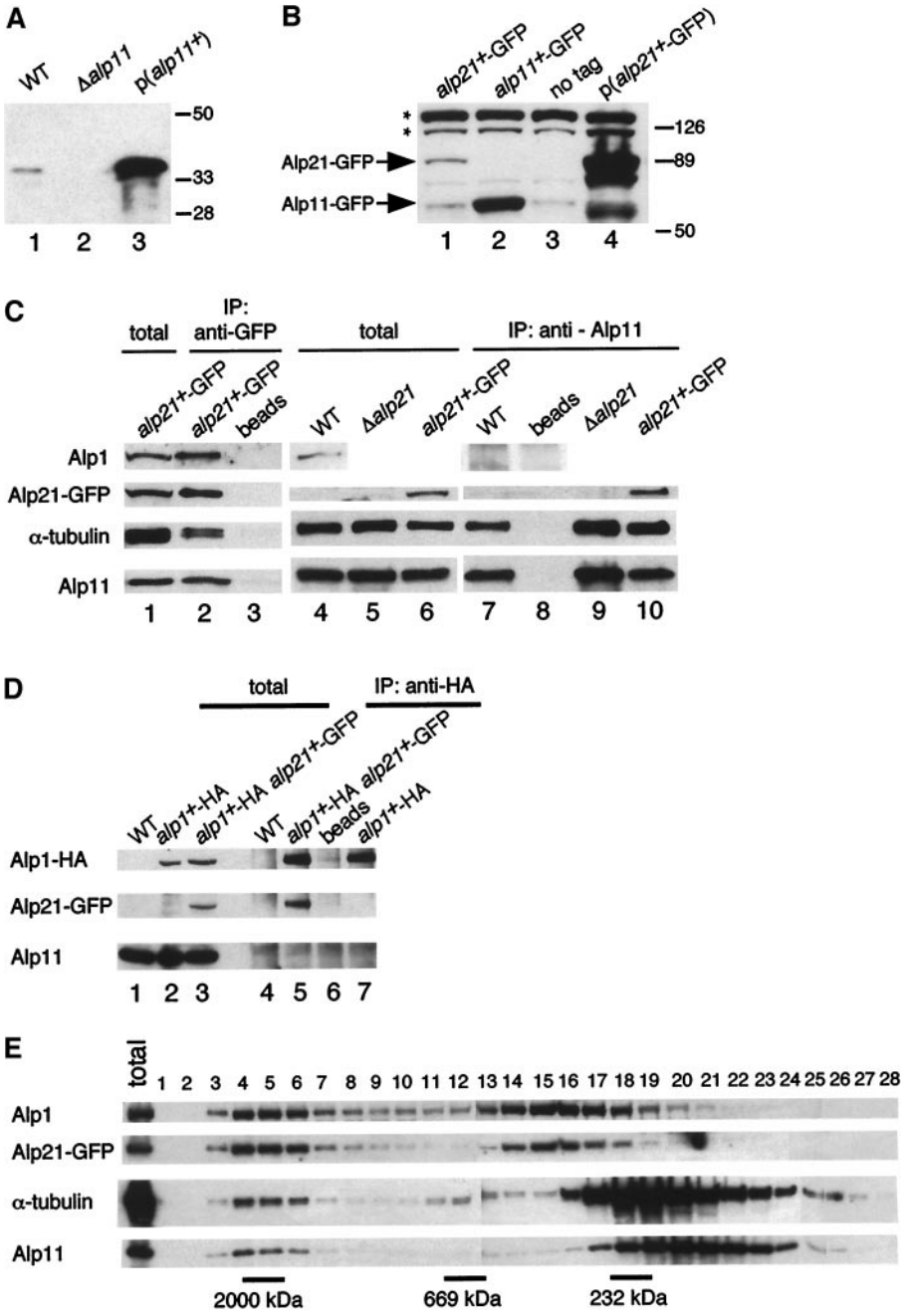

Figure 4. Interaction and gel filtration analysis of cofactors and tubulins. (A) Identification of the *alp11*$^+$ gene product. Immunoblotting was performed with anti-Alp11 antibody against cell extracts prepared from wild type (lane 1), Δ*alp11* (lane 2; PR9), or wild type carrying multicopy *alp11*$^+$ (lane 3). The positions of size markers (kilodaltons) are shown on the right. (B) Identification of the *alp21*$^+$*-GFP* gene product. Immunoblotting was performed with anti-GFP antibody against cell extracts prepared from wild type (lane 3), strains containing integrated *alp11*$^+$*-GFP* (lane 2; PR11) or *alp21*$^+$*-GFP* (lane 1; PR12), or the strain carrying multicopy *alp21*$^+$*-GFP* (lane 4). Asterisks show nonspecific bands. (C and D) Interaction among cofactors and α-tubulin. Cell extracts were prepared from wild type (C, lanes 4 and 7, and D, lanes 1 and 4), a strain containing integrated *alp21*$^+$*-GFP* (C, lanes 1, 2, 3, 6, 8, and 10; PR12), Δ*alp21* containing pDB-*alp1*$^+$ (C, lanes 5 and 9; PR10), a strain containing integrated *alp1*$^+$*-HA* (D, lanes 2 and 7; PR15), or a strain containing *alp1*$^+$*-HA* and *alp21*$^+$*-GFP* (D, lanes 3, 5, and 6; PR16), and immunoprecipitation (shown as IP) was performed with anti-GFP antibody (C, lane 2), anti-Alp11 antibody (C, lanes 7, 9, and 10), anti-HA antibody (D, lanes 4, 5, and 7), or mock treatment (C, lanes 3 and 8, and D, lane 6). Precipitated proteins were detected with anti-Alp1, anti-GFP, anti-α-tubulin, anti-Alp11, and anti-HA antibodies. Total cell extracts (corresponding to 1/30th the amount used for immunoprecipitation) were also run (C, lanes 1, 4, 5, and 6, and D, lanes 1, 2, and 3). (E) Gel filtration chromatography. Soluble extracts from a strain containing integrated *alp21*$^+$*-GFP* (PR18) were separated through a Superose-6 column, and fractions were analyzed by immunoblotting with the antibodies shown. Total extract (10 μg) was run on the far left panel. The positions of size markers (232, 669, and 2000 kDa) are also shown.

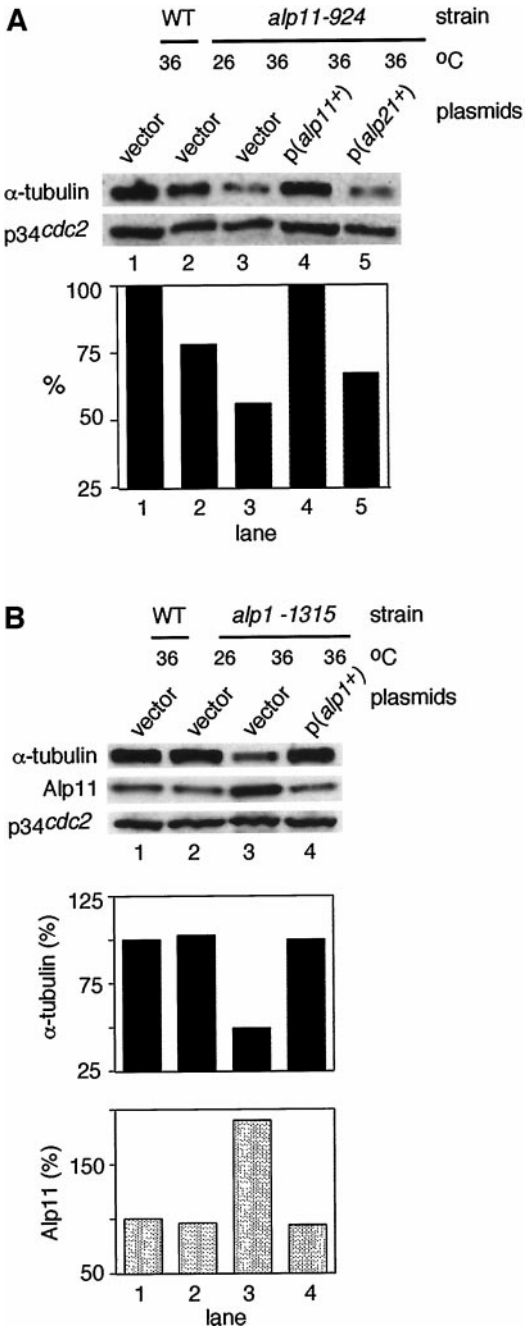

Figure 5. The level of α-tubulin decreases in the *alp11* and *alp1* mutants. (A) The level of α-tubulin in the *alp11* mutant. Wild-type (lane 1) and *alp11-924* (lanes 2–5) strains containing vector (lanes 1–3), pREP-*alp11*$^+$ (lane 4), or pREP-*alp21*$^+$ (lane 5) were grown at 26°C in the absence of thiamine for 12 h (lane 2), shifted to 36°C, and incubated for 8 h (lanes 1 and 3–5). Immunoblotting was performed with the indicated antibodies. (B) The level of α-tubulin and Alp11^B in the *alp1* mutant. Wild-type (lane 1) and *alp1-1315* (lanes 2–4; DH2-8D) strains containing vector (lanes 1–3) or pDB-*alp1*$^+$ (lane 4) were grown and immunoblotted with the indicated antibodies. Densitometric calibration of the data is shown in the lower panels in A and B. The relative levels of α-tubulin and Alp11^B were measured with the level of Cdc2 as an internal control.

A similar analysis was performed with a temperature-sensitive *alp1* strain. As in the *alp11* mutant, it was found that the level of α-tubulin also significantly decreased in this mutant (Figure 5B, lanes 1 and 3). Conversely, Alp11^B levels increased significantly (190%) in this mutant. These defects were reversed by the introduction of the wild-type *alp1*$^+$ gene on plasmids (Figure 5B, lane 4). In contrast, the level of β-tubulin appeared unchanged in both *alp1* and *alp11* mutants (our unpublished results). These results suggested that unfolded α-tubulin monomers are unstable in the absence of Alp11^B or Alp1^D function and that Alp1^D negatively regulates the level of Alp11^B.

Ectopic Overexpression of alp11$^+$ Is Toxic and Rescued by Coexpression of the α-Tubulin Gene

Our previous results showed that ectopic overexpression of cofactor D–encoding *alp1*$^+$ was lethal (Hirata *et al.*, 1998), suggesting that perturbation of the stoichiometry of cofactors is toxic to the cell. To examine the phenotypes arising from overproduction of fission yeast cofactors B and E, the entire ORFs of the *alp11*$^+$ and *alp21*$^+$ genes were inserted into plasmids containing the thiamine-repressible *nmt1* promoter (pREP-*alp11*$^+$ and pREP-*alp21*$^+$). It was found that *alp11*$^+$, but not *alp21*$^+$, was lethal when ectopically overexpressed (Figure 6A).

Cells in which *alp11*$^+$ was overexpressed showed elongated morphology, and prolonged incubation resulted in bent or abnormal morphology, which was similar to that of temperature-sensitive *alp11-924*. Staining of these *alp11*$^+$-overexpressing cells with anti-tubulin antibody revealed that in the majority of cells no intact MTs were present; instead, uniform cytoplasmic staining was evident (Figure 6B, left). This result showed that an excess amount of Alp11^B abrogates MT structure and function. If this toxicity were due to the binding and absorption of free α-tubulin, co-overexpression of an α-tubulin–encoding gene might rescue the lethality. That was indeed the case. As shown in Figure 6C, cells coexpressing *alp11*$^+$ and *atb2*$^+$ (α2-tubulin; Adachi *et al.*, 1986), but not *nda3*$^+$ (β-tubulin; Hiraoka *et al.*, 1984), were viable. This result suggested that the lethality of excess Alp11^B is attributable to the absorption of free α-tubulin molecules.

The Glycine-rich Domain Is Dispensable but Required for the Efficient Interaction between Alp11^B and Free α-Tubulins, and Also Alp21^E

The Alp11^B glycine-rich domain locates in the C-terminal region (Figure 2, A and B, CLIP-170 domain, amino acids 166–216). To test whether this domain is essential for Alp11^B function, DNA fragments encoding N-terminal (amino acids 1–163) or C-terminal (amino acids 131–234) portions of Alp11^B were subcloned into plasmids under control of the *nmt1* promoter (pREP-alp11$_{1–163}$ and pREP-alp11$_{131–234}$, respectively), and suppression over Δ*alp11* was examined. As shown in Figure 7A, pREP-alp11$_{1–163}$, but not pREP-alp11$_{131–234}$, was capable of suppressing the growth defects of Δ*alp11*. It is of note that, unlike intact Alp11, neither Alp11$_{1–163}$ nor Alp11$_{131–234}$ was toxic when ectopically overproduced. To confirm the dispensability of the CLIP-170 domain, the C-terminal region was deleted in one of the chromosomal *alp11*$^+$ genes of a diploid strain (truncated

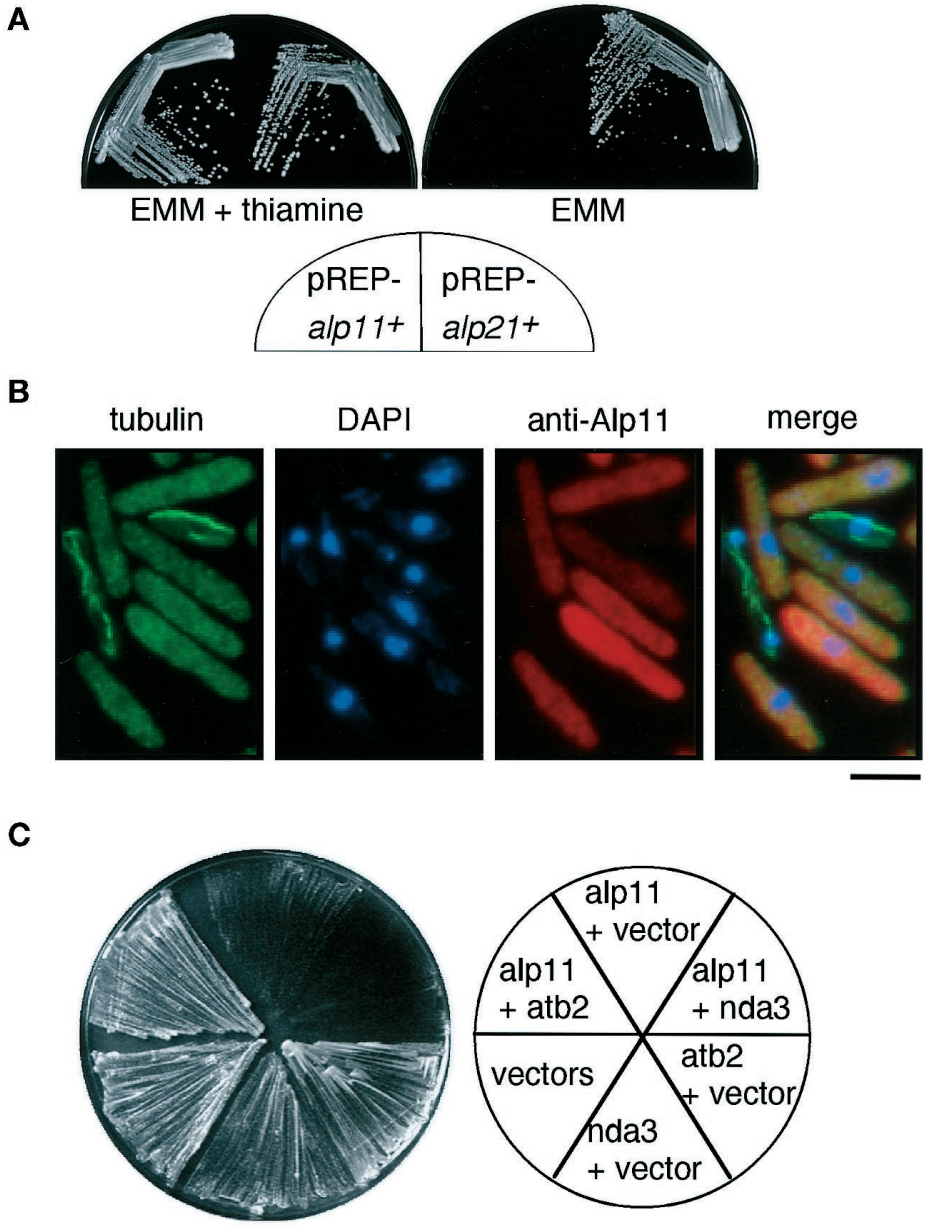

Figure 6. Ectopic overexpression of *alp11*$^+$. (A) Lethal overexpression of *alp11*$^+$. Cells containing pREP-*alp11*$^+$ (left on each plate) or pREP-*alp21*$^+$ (right) were streaked on minimal plates in the presence (left plate) or absence (right) of thiamine and incubated for 3 d at 26°C. Overproduction of each protein has been checked by immunoblotting. (B) Microtubule structures in *alp11*$^+$-overexpressing cells. Cells containing pREP-*alp11*$^+$ were grown in the absence of thiamine for 23 h and processed for immunofluorescence microscopy using anti-α-tubulin antibody and anti-Alp11 antibody. Chromosomal DNA was stained with DAPI. The merged image is shown on the right. Note that no MTs were observed in most cells in which Alp11^B was overexpressed and that the presence of MTs correlates with lower Alp11^B levels. Bar, 10 μm. (C) Suppression of lethal overexpression of *alp11*$^+$ by coexpression of *atb2*$^+$. Wild-type cells were doubly transformed with the following plasmids: two empty vectors (pREP1 and pREP2), a vector plus pREP-*alp11*$^+$, a vector plus pREP2-*atb2*$^+$, a vector plus pREP2-*nda3*$^+$, pREP-*alp11*$^+$ plus pREP2-*atb2*$^+$, or pREP-*alp11*$^+$ plus pREP2-*nda3*$^+$. Transformants were streaked onto minimal media without thiamine and incubated for 3 d at 29°C.

Alp11 protein was designated Alp11$_{1–163}$). It was found that the growth of haploid cells containing Alp11$_{1–163}$ was indistinguishable from that of wild-type cells (Figure 7B).

As the CLIP-170 domain appears to be involved in MT binding in other proteins (Rickard and Kreis, 1996) and Alp11^B interacts with α-tubulin, the role of the CLIP-170 domain in the binding of α-tubulin was addressed. Cell extracts were prepared from a strain in which this domain was deleted (Alp11$_{1–163}$), and immunoprecipitation was performed using anti-Alp11 antibody. It was found that the binding between Alp11$_{1–163}$ and α-tubulins was greatly diminished (Figure 7C, lane 5). Furthermore, Alp11$_{1–163}$ lost the ability to associate stably not only with α-tubulin but also with Alp21^E (Figure 4D, lane 3). Thus, the CLIP-170 domain of Alp11^B is dispensable for the essential function of Alp11^B but confers a lethal interfering effect when overproduced and, importantly, is required for the efficient binding between Alp11^B and α-tubulin, and also Alp21^E. In other words, the interaction between Alp11^B and both Alp21^E and α-tubulin is dependent on the CLIP-170 domain.

The Cellular Localization of Alp11^B

The cellular localization of Alp11^B was examined in detail by fluorescence microscopy with the use of specific antibodies and GFP. Immunofluorescence microscopy in wild-type cells with anti-Alp11 showed punctate cytoplasmic staining (Figure 8A, left). The staining is specific, as Δ*alp11* cells

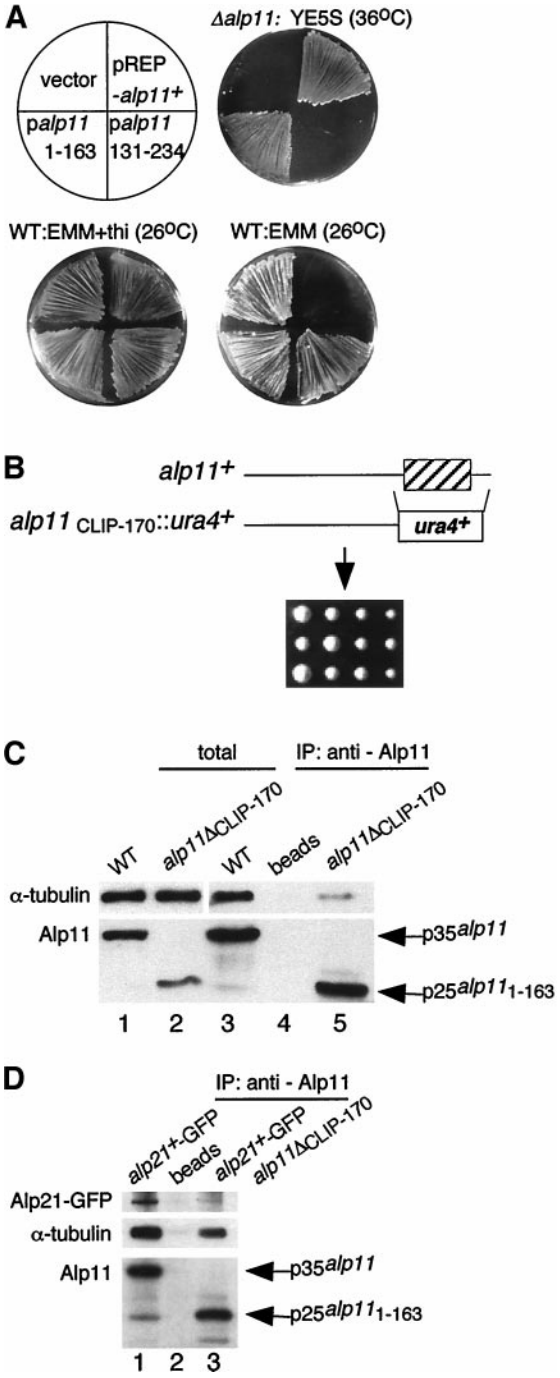

Figure 7. The function of the conserved glycine-rich domain of Alp11^B. (A) Suppression of the growth defects of *alp11*-deleted cells. A Δ*alp11* strain (PR9) was transformed with plasmids containing the *alp11*$^+$ gene (pREP-*alp11*$^+$; top right), the C-terminal region (pREP-alp11$_{131–234}$; bottom right), the N-terminal region (pREP-alp11$_{1–163}$; bottom left), or a vector (top left), streaked on rich medium at 36°C (top right plate) or minimal medium in the absence (lower right plate) or presence (lower left plate) of thiamine at 26°C, and incubated for 4 d. (B) The glycine-rich region of Alp11^B is dispensable. The CLIP-170 domain is shown as a hatched box (top). Tetrad analysis was performed with a heterozygous diploid in which one copy of the *alp11* genes was truncated at amino acid 163 (bottom; PR13). (C) The glycine-rich domain is important for efficient binding between Alp11^B and α-tubulin. Immunoprecipitation (IP) was performed with anti-Alp11 antibody (lanes 3 and 5) or mock treatment (lane 4) against cell extracts prepared from wild-type (lanes 1, 3, and 4) or a strain containing Alp11$_{1–163}$ (shown as *alp11*ΔCLIP-170) (lanes 2 and 5; PR14). Total extracts were also run (lanes 1 and 2). (D) The glycine-rich domain is required for association between Alp11^B and Alp21^E. A strain containing Alp11$_{1–163}$ and integrated *alp21*$^+$*-GFP* (PR17) was used for immunoprecipitation with anti-Alp11 antibody, followed by immunoblotting with anti-GFP, anti-α-tubulin, and anti-Alp11 antibodies.

showed no signals (Figure 8A, right). Alp21^E also showed cytoplasmic staining when overproduced (our unpublished results). A similar pattern was observed with GFP staining in cells carrying multicopy *alp11*$^+$-GFP. We also examined the localization of Alp11$_{1–163}$ and again observed only cytoplasmic staining (Figure 8B). The same cytoplasmic staining was also observed in cells expressing Alp11$_{131–234}$-GFP (Figure 8C). These data show that Alp11^B localizes to the cytoplasm and that, in the case of this protein, the CLIP-170 domain appears to play no role in MT binding.

Increased Level of Alp1^D Is Sufficient to Rescue the Loss of Alp11^B or Alp21^E

To establish the functional order of cofactors in the tubulin-folding pathway in vivo, multicopy plasmids containing cofactor homologues were introduced into each *alp* mutant. It transpired that the temperature-sensitive growth defects of an *alp11* deletion strain were suppressed by multicopy plasmids containing *alp1*$^+$, *alp11*$^+$, or *alp21*$^+$ (Figure 9A). An identical suppression result was obtained when a diploid heterozygous for *alp11* was used (Table 3). Second, the *alp21* disruption was rescued by multicopy plasmids containing *alp1*$^+$ or *alp21*$^+$ but not *alp11*$^+$ (Figure 9B, Table 3).

Finally, temperature-sensitive *alp1* mutations were not suppressed by plasmids containing either *alp11*$^+$ or *alp21*$^+$ (Figure 9C). Nor could the *alp1* deletion be rescued by overexpression of either of these two genes (Table 3). Also, multicopy plasmids containing the α-tubulin–encoding gene (*atb2*$^+$) are not capable of complementing temperature-sensitive *alp1* or *alp11* mutants. These results indicate that the essential *alp11*$^+$ gene becomes dispensable when an excess level of Alp21^E or Alp1^D is supplied, and similarly that *alp21*$^+$ is nonessential when Alp1^D exists in a larger amount. Therefore, the cofactor homologues appear to operate in a linear manner (Alp11^B-Alp21^E-Alp1^D) in the biogenesis of MTs.

DISCUSSION

An important notion arising from the present study is the existence of a functional hierarchy among cofactors. Biochemical analysis in mammalian systems showed cofactors E and D to have parallel and indispensable roles in the folding of α- and β-tubulins, respectively, in which these cofactors are proposed to form a quadruple complex with α- and β-tubulin upon convergence of the two distinct folding pathways (Tian *et al.*, 1996, 1997). A crucial finding from our

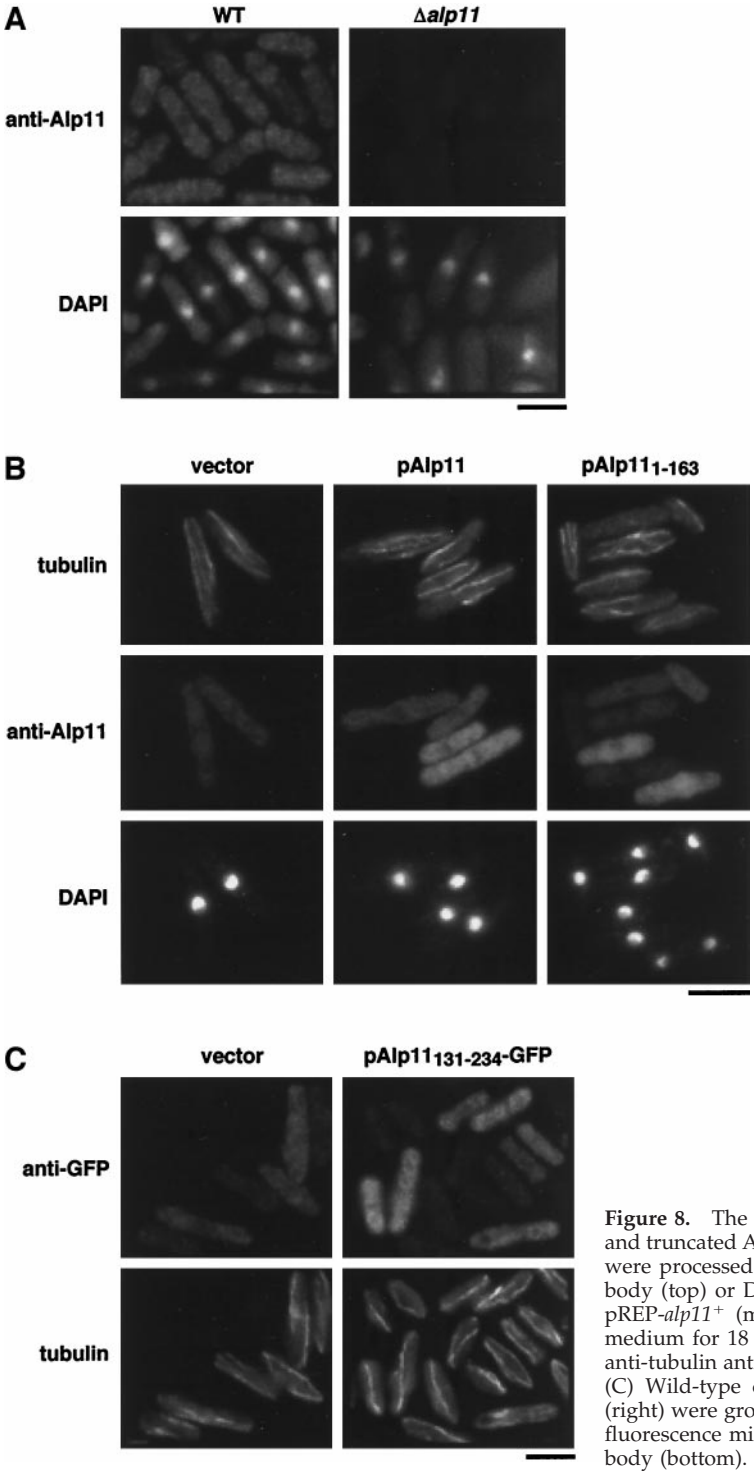

Figure 8. The cellular localization of Alp11. Immunolocalization of Alp11^{B} and truncated Alp11^{B} proteins. (A) Wild-type (WT; left) or Δ*alp11* (right) cells were processed for immunofluorescence microscopy using anti-Alp11 antibody (top) or DAPI (bottom). (B) Wild-type cells containing a vector (left), pREP-*alp11*$^{+}$ (middle), or pREP-alp11$_{1–163}$ (right) were grown in minimal medium for 18 h and processed for immunofluorescence microscopy using anti-tubulin antibody (top), anti-Alp11 antibody (middle), or DAPI (bottom). (C) Wild-type cells containing a vector (left) or pREP42-GFP-alp11$_{131–234}$ (right) were grown in minimal medium for 18 h and processed for immunofluorescence microscopy using anti-GFP antibody (top) or anti-tubulin antibody (bottom). Bars, 10 μm.

study is the bypass of the Alp21^{E} requirement by overproduction of Alp1^{D} (Figure 10A). This situation has never been observed in in vitro tubulin-folding reactions, in which cofactor E is absolutely required for the formation of correctly folded α/β-tubulin heterodimers. It is likely that Alp1^{D} is required for the folding of both α- and β-tubulins, as suggested (Tian *et al.*, 1996, 1997); however, the folding of α-tubulin may be more complex than that of β-tubulin. At present, the importance of the in vivo β-tubulin–folding pathway remains to be determined. Analysis of the recently

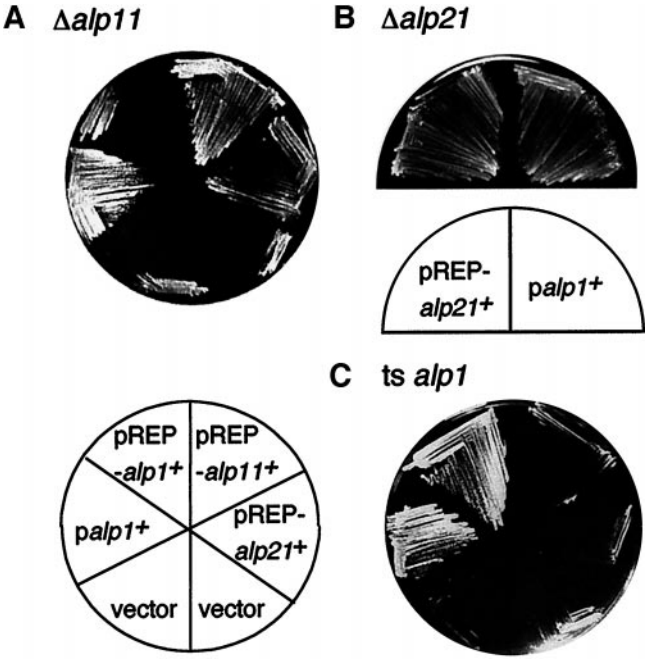

Figure 9. Functional suppression. (A) Suppression of the *alp11* mutation. Δ*alp11* (PR9) was transformed with the plasmids shown, and transformants were streaked on a minimal plate containing thiamine and incubated at 36°C for 3 d. pREP-*alp1*$^+$ was not capable of suppressing the *alp11* mutant under these conditions (repressed). (B) Suppression of the *alp21* disruptant. A diploid heterozygous for *alp21* (PR8) was transformed with various plasmids, and random spore analysis was performed. Leu$^+$Ura$^+$ colonies were streaked on minimal plates containing thiamine and incubated at 30°C for 3 d. Haploid Δ*alp21* strains containing pREP-*alp21*$^+$ (left) or pDB-*alp1*$^+$ (right; PR10) are shown. (C) Suppression of the *alp1* mutation. *alp1-1315* (DH2-8D) was transformed with the plasmids indicated and streaked.

identified *S. pombe* homologue of cofactor A (P.A. Radcliffe and T. Toda, unpublished results) and the further analysis of Alp1^D should clarify this point.

Table 3. Suppression analysis among cofactor homologs

	Gene on plasmid			
Deleted gene	Vector	*alp11*$^+$	*alp21*$^+$	*alp1*$^+$
Δ*alp11*	−	+	+	+
Δ*alp21*	−	−	+	+
Δ*alp1*	−	−	−	+

Heterozygous diploids (ME1, PR7, and PR8; see Table 1) were transformed with a vector (pREP1) or multicopy plasmids containing individual genes. Diploid transformants were allowed to sporulate, and free spores were spread on minimal plates supplemented with histidine and adenine to select viable Leu$^+$Ura$^+$ haploid colonies. (+) We obtained Leu$^+$Ura$^+$ haploid cells that contain the deleted gene carrying the indicated plasmids; (−) we failed to obtain Ura$^+$ haploid.

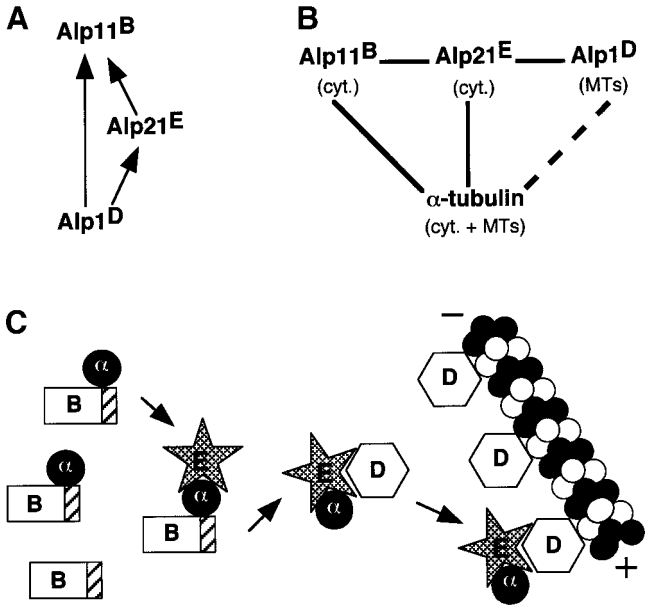

Figure 10. Model for the sequential operation of cofactors in microtubule biogenesis. (A) Genetic suppression. Arrows (X→Y) indicate that a high level of "X" suppresses loss of "Y" function. (B) Physical interaction. Solid lines (X—Y) indicate interaction between "X" and "Y" by immunoprecipitation. The dashed line indicates interaction by in vitro MT-binding assay (Hirata *et al.*, 1998). cyt., localization in cytoplasm; MTs, colocalization with MTs. (C) A schematic model of cofactor functions. Cofactor B (Alp11) captures α-tubulin molecules released from chaperonins (not shown) and maintains a reservoir of free α-tubulins, which in its absence become unstable. The α-tubulin–binding CLIP-170 domain is depicted by hatched boxes. Cofactor E (Alp21) binds cofactor B, α-tubulin, and cofactor D (Alp1), perhaps via its central region of leucine-rich repeats. It acts as a mediator transporting α-tubulin between cofactors B and D. Interaction between cofactors B and E might be indirect, possibly through α-tubulin. A subpopulation of cofactor D colocalizes with microtubules and delivers α-tubulin (or α/β-heterodimer) to growing microtubule tips.

The most simple explanation of our data would be that cofactors constitute a functionally linear pathway in vivo: Alp11^B-Alp21^E-Alp1^D (Figure 10B), although alternative models remain possible. For example, Alp21^E and Alp1^D may function together rather than in a sequential manner. Immunoprecipitation and gel filtration analysis support this latter possibility. The quadruple complex (Eα Dβ) may exist transiently in the cell, but this complex formation is not essential for MT biogenesis, and Alp21^E is dispensable if Alp1^D is supplied in excess in fission yeast. Of most importance is the genetic epistasis in which multicopy plasmids containing *alp1*$^+$ suppress *alp21*-deleted cells. In line with our data, in budding yeast partial suppression of the benomyl sensitivity of mutations in *PAC2* (encoding cofactor E homologue) by multicopy *CIN1* (cofactor D homologue) has been reported (Hoyt *et al.*, 1997). Because of the difference between the mammalian and yeast systems, a direct comparison might be difficult. Further work will be required to clarify the mode of action of these cofactors in tubulin-folding pathways.

Distinct Roles of Cofactors in MT Biogenesis

Our analysis is consistent with the idea that Alp11^B captures newly synthesized α-tubulin molecules that are released from chaperonins and maintains a reservoir of partially folded α-tubulin in the cell (Figure 10C) (Tian *et al.*, 1997; Feierbach *et al.*, 1999). It binds and perhaps protects unfolded α-tubulin from degradation, although the stable binding between Alp11^B and α-tubulin appears not to be essential for Alp11^B function. Alp21^E, which interacts with both Alp11^B and Alp1^D as well as α-tubulin, may function as a mediator that transfers α-tubulin from the pool maintained by Alp11^B to Alp1^D. It should be noted that interaction between Alp11^B and Alp21^E requires the CLIP-170 domain of Alp11^B. This suggests either that the CLIP-170 domain has a dual binding capacity for both α-tubulin and Alp21^E or that interaction between Alp11^B and Alp21^E is indirect and occurs via α-tubulin. Although we cannot rule out the former possibility, suppression of lethal Alp11^B overproduction by simultaneous expression of α-tubulin alone suggests that Alp11^B and Alp21^E interact indirectly. Some population of Alp1^D colocalizes with MTs in vivo and cosediments with polymerized porcine brain MTs in vitro (Hirata *et al.*, 1998). This behavior is unique among the cofactors; as shown in this study, we failed to see colocalization of either Alp11^B or Alp21^E with MTs. It is possible that cofactor D functions to deliver a cargo of α/β-heterodimers to the "plus" end of MTs via MTs (see Figure 10C). Cofactor D may also function to preserve MT integrity. We note that Alp1^D and mammalian cofactor D contain two repeats of HEAT motifs that are involved in protein-protein interactions (Andrade and Bork, 1995; Groves *et al.*, 1999).

That cells have such an elaborate mechanism for the folding and transport of tubulin molecules indicates that an abundance of free tubulin molecules is likely to be deleterious. This implies that flux through the folding pathway, especially the later stages, may be tightly coordinated with the changing needs of the cell. In this way, the cell is able to quickly mobilize this supply to maximize heterodimer availability at times of high demand. In this context, it is likely that activities of cofactors, either quantitatively or qualitatively, are regulated during the cell cycle or developmental stage. Up-regulation of Alp11^B in the temperature-sensitive *alp1* mutant suggests that a negative feedback mechanism may exist that regulates the relative abundance of each cofactor.

Regulation of Tubulin Levels by Cofactors

Ectopic overproduction of Alp11^B, like that of Alp1^D (Hirata *et al.*, 1998), is toxic. We note that this toxic effect is enhanced at lower temperatures (26°C). Observation of overproducing cells revealed phenotypes similar to those of *alp11* mutants, including polarity defects and loss of MTs. This indicates that severe perturbation of Alp11^B levels results in a lethal shortage of correctly folded α-tubulin molecules. It is likely that excess Alp11^B captures α-tubulin irreversibly, thereby inhibiting subsequent reactions by Alp21^E. Consistent with this effect, an increased level of α-tubulin in Alp11^B-overproducing cells rescues the lethality. The endogenous level of Alp11^B in the cell is much higher (>10-fold) than that of Alp21^E, which would correlate well with the roles of cofactors B and E as a reservoir and as a mediator, respectively, of free α-tubulin.

As shown in this study, the steady-state levels of α-tubulin decrease substantially in the temperature-sensitive *alp1* and *alp11* mutants. In agreement with this phenomenon, in in vitro systems perturbation of the stoichiometry of cofactors results in the instability of tubulin molecules (Tian *et al.*, 1997). As cofactor D is involved in the folding of both α- and β-tubulins (Tian *et al.*, 1996, 1997), one might expect that in *alp1* mutants the levels of β-tubulins also decrease, but this was not the case. It appears that the physical states of α- and β-tubulin in the absence of Alp1^D function differ in terms of protein stability. This implies that α-tubulin is the major target for regulation of the level of free tubulin pools via cofactors. It may also explain why an excess of β-tubulin is more harmful to the cell than an excess of α-tubulin (Hiraoka *et al.*, 1984; Toda *et al.*, 1984).

Structural Conservation and Diversity of Cofactor E

The LRR motif is a conserved structural feature present in cofactor E from all species. Although mammalian cofactor E contains the CLIP-170 domain (as does the budding yeast homologue Pac2; Hoyt *et al.*, 1997; Tian *et al.*, 1997), fission yeast Alp21^E does not. It is possible that fission yeast has evolved differently from other organisms, such that Alp21^E has lost a crucial function that both budding yeast Pac2 and mammalian cofactor E retain. Alternatively, as in the case of Alp11^B, the CLIP-170 domain in cofactor E may play an accessory role in its interaction with α-tubulin, with the LRRs crucial for the function of cofactor E. Thus, it is possible that the CLIP-170 domain has been lost from Alp21^E during evolution because it is not essential.

Function of the Glycine-rich (CLIP-170) Domain in Binding Tubulin and MTs

We have shown here that the CLIP-170 domain of Alp11^B is required for the efficient binding of α-tubulin by the cofactor. It has been proposed and generally assumed that the CLIP-170 domain is a MT-binding motif (Riehemann and Sorg, 1993; Rickard and Kreis, 1996). There is solid evidence to support the notion that all of the CLIP-170-domain–containing proteins are indeed involved in MT-related processes; however, not all of these proteins are MT-associated proteins. For example, p150^{Glued} and CLIP-170 do not always colocalize with MTs in the cell (Dujardin *et al.*, 1998; Robinson *et al.*, 1998). Also, vertebrate cofactors B and E and fission yeast Alp11^B are not MT-binding proteins (Tian *et al.*, 1997; this study). It is nonetheless possible that MT binding of Alp11^B was not detected for some technical reason or that only a subpopulation of Alp11^B colocalizes with MTs. In budding yeast, Alf1^B has been shown to localize with MTs when overexpressed (Feierbach *et al.*, 1999). Alternatively, the CLIP-170 domain is not sufficient for MT binding but is an archetypal motif for α-tubulin binding, as has been suggested (Lewis *et al.*, 1997).

In line with this idea, it has recently been shown that budding yeast Alf1^B also binds free α-tubulin (Feierbach *et al.*, 1999). It is possible that the CLIP-170-domain–containing proteins that bind MTs require additional amino acid sequences other than the CLIP-170 domain to ensure MT

binding. For instance, many of these CLIP-170-domain–containing proteins consist of regions with coiled-coil structures and/or those rich in basic amino acid residues adjacent to the CLIP-170 domain (Rickard and Kreis, 1996). Alternatively, minor evolutionary modification of this domain or adjacent regions may have conferred the ability to discriminate between the various forms of α-tubulin in the cell (i.e., monomers and MTs). Detailed domain analysis of these proteins or domain-swapping experiments between different proteins will be required for further clarification of the molecular role of the CLIP-170 domain.

Note added in proof

While this paper was under review, Grishchuk and McIntosh published the paper on Alp21 (called Sto1, Sto1p a fission yeast protein similar to tubulin folding Cofactor E, plays an essential role in mitotic microtubules assembly [J Cell Sci. (1999). *112*, 1979–1988], in which the authors show A1p21 also localizes in the cytoplasm.

ACKNOWLEDGMENTS

We thank Drs. Keith Gull, Kenneth Sawin, and Hiroyuki Yamano for providing materials used in this study. We are grateful to Drs. Ekaterina L. Grishchuk, J. Richard McIntosh, and Masayuki Yamamoto for sharing results before publication. We thank Drs. Chris Norbury and Itziar Ochotorena for instruction and help in gel filtration. We thank Drs. Jacqueline Hayles, Paul Nurse, and Graham Warren for critical reading of the manuscript and useful suggestions. This article is dedicated to the memory of Kazuhiko Umesono.

REFERENCES

Adachi, Y., Toda, T., and Yanagida, M. (1986). Differential expression of essential and nonessential α-tubulin genes in *Schizosaccharomyces pombe*. Mol. Cell. Biol. *6*, 2168–2178.

Andrade, M.A., and Bork, P. (1995). HEAT repeats in the Huntington's disease protein. Nat. Genet. *11*, 115–116.

Archer, J.E., Vega, L.R., and Solomon, F. (1995). Rbl2p, a yeast protein that binds to β-tubulin and participates in microtubule function in vivo. Cell *82*, 425–434.

Bachurski, C., Theodorakis, N., Coulson, R.M., and Cleveland, D.W. (1994). An amino-terminal tetrapeptide specifies cotranslational degradation of β-tubulin but not α-tubulin mRNA. Mol. Cell. Biol. *14*, 4076–4086.

Bähler, J., Wu, J., Longtine, M.S., Shah, N.G., McKenzie, A., III, Steever, A.B., Wach, A., Philippsen, P., and Pringle, J.R. (1998). Heterologous modules for efficient and versatile PCR-based gene targeting in *Schizosaccharomyces pombe*. Yeast *14*, 943–951.

Belmont, L.D., and Mitchison, T.J. (1996). Identification of a protein that interacts with tubulin dimers and increases the catastrophe rate of microtubules. Cell *84*, 623–631.

Bilbe, G., *et al.* (1992). Restin: a novel intermediate filament-associated protein highly expressed in the Reed-Sternberg cells of Hodgkin's disease. EMBO J. *11*, 2103–2113.

Craven, R.A., Griffiths, D.J.F., Sheldrick, K.S., Randall, R.E., Hagan, I.M., and Carr, A.M. (1998). Vectors for the expression of tagged proteins in *Schizosaccharomyces pombe*. Gene *221*, 59–68.

Dujardin, D., Wacker, U.I., Moreau, A., Schroer, T.A., Rickard, J.E., and De Mey, J.R. (1998). Evidence for a role of CLIP-170 in the establishment of metaphase chromosome alignment. J. Cell Biol. *141*, 849–862.

Feierbach, B., Nogales, E., Downing, K.H., and Stearns, T. (1999). Alf1p, a CLIP-170 domain-containing protein, is functionally and physically associated with α-tubulin. J. Cell Biol. *144*, 113–124.

Gao, Y., Melki, R., Walden, P.D., Lewis, S.A., Ampe, C., Rommelaere, H., Vandekerckhove, J., and Cowan, N.J. (1994). A novel cochaperonin that modulates the ATPase activity of cytoplasmic chaperonin. J. Cell Biol. *125*, 989–996.

Gao, Y., Thomas, J.O., Chow, R.L., Lee, G.H., and Cowan, N.J. (1992). A cytoplasmic chaperonin that catalyzes β-actin folding. Cell *69*, 1043–1050.

Gao, Y., Vainberg, I.E., Chow, R.L., and Cowan, N.J. (1993). Two cofactors and cytoplasmic chaperonin are required for the folding of α- and β-tubulin. Mol. Cell. Biol. *13*, 2478–2485.

Geissler, S., Siegers, K., and Schiebel, E. (1998). A novel protein complex promoting formation of functional α- and γ-tubulin. EMBO J. *17*, 952–966.

Gill, S.R., Schroer, T.A., Szilak, I., Steuer, E.R., Sheetz, M.P., and Cleveland, D.W. (1991). Dynactin, a conserved, ubiquitously expressed component of an activator of vesicle motility mediated by cytoplasmic dynein. J. Cell Biol. *115*, 1639–1650.

Gonzalez-Garay, M.L., and Cabral, F. (1996). alpha-Tubulin limits its own synthesis: evidence for a mechanism involving translational repression. J. Cell Biol. *135*, 1525–1534.

Groves, M.R., Hanlon, N., Turowski, P., Hemmings, B.A., and Barford, D. (1999). The structure of the protein phosphatase 2A PR65/A subunit reveals the conformation of its 15 tandemly repeated HEAT motifs. Cell *96*, 99–110.

Hiraoka, Y., Toda, T., and Yanagida, M. (1984). The *NDA3* gene of fission yeast encodes β-tubulin: a cold-sensitive *nda3* mutation reversibly blocks spindle formation and chromosome movement in mitosis. Cell *39*, 349–358.

Hirata, D., Masuda, H., Eddison, M., and Toda, T. (1998). Essential role of tubulin-folding cofactor D in microtubule assembly and its association with microtubules in fission yeast. EMBO J. *17*, 658–666.

Hirokawa, N. (1998). Kinesin and dynein superfamily proteins and the mechanism of organelle transport. Science *279*, 519–526.

Horio, T., and Hotani, H. (1986). Visualization of the dynamic instability of individual microtubules by dark-field microscopy. Nature *321*, 605–607.

Hoyt, M.A., Macke, J.P., Roberts, B.T., and Geiser, J.R. (1997). *Saccharomyces cerevisiae PAC2* functions with *CIN1*, *2* and *4* in a pathway leading to normal microtubule stability. Genetics *146*, 849–857.

Hoyt, M.A., Stearns, T., and Botstein, D. (1990). Chromosome instability mutants of *Saccharomyces cerevisiae* that are defective in microtubule-mediated processes. Mol. Cell. Biol. *10*, 223–234.

Hyman, A.A., and Karsenti, E. (1996). Morphogenetic properties of microtubules and mitotic spindle assembly. Cell *84*, 401–410.

Kobe, B., and Deisenhofer, J. (1994). The leucine-rich repeat: a versatile binding motif. Trends Biochem. Sci. *19*, 415–421.

Kominami, K., Seth-Smith, H., and Toda, T. (1998). Apc10 and Ste9/Srw1, two regulators of the APC-cyclosome, as well as the CDK inhibitor Rum1 are required for G_1 cell cycle arrest in fission yeast. EMBO J. *17*, 5388–5399.

Kubota, H., Hynes, G., Carne, A., Ashworth, A., and Willison, K. (1994). Identification of six *Tcp-1*-related genes encoding divergent subunits of the TCP-1-containing chaperonin. Curr. Biol. *4*, 89–99.

Lewis, S.A., Tian, G., and Cowan, N.J. (1997). The α- and β-tubulin folding pathways. Trends Cell Biol. *7*, 479–484.

Lewis, V.A., Hynes, G.M., Zheng, D., Saibil, H., and Willison, K. (1992). T-complex polypeptide-1 is a subunit of a heteromeric particle in the eukaryotic cytosol. Nature *358*, 249–252.

Li, H.P., Liu, Z.M., and Nirenberg, M. (1997). Kinesin-73 in the nervous system of *Drosophila* embryos. Proc. Natl. Acad. Sci. USA *94*, 1086–1091.

Llosa, M., Aloria, K., Campo, R., Padilla, R., Avila, J., Sánchez-Pulido, L., and Zabala, J.C. (1996). The β-tubulin monomer release factor (p14) has homology with a region of the DnaJ protein. FEBS Lett. *397*, 283–289.

Mandelkow, E., and Mandelkow, E.-M. (1995). Microtubules and microtubule-associated proteins. Curr. Opin. Cell Biol. *7*, 72–81.

Maundrell, K. (1990). *nmt1* of fission yeast. J. Biol. Chem. *265*, 10857–10864.

McNally, F.J., and Vale, R.D. (1993). Identification of katanin, an ATPase that severs and disassembles stable microtubules. Cell *75*, 419–429.

Melki, R., Rommelaere, H., Leguy, R., Vandekerckhove, J., and Ampe, C. (1996). Cofactor A is a molecular chaperone required for β-tubulin folding: functional and structural characterization. Biochemistry *35*, 10422–10435.

Melki, R., Vainberg, I.E., Chow, R.L., and Cowan, N.J. (1993). Chaperonin-mediated folding of vertebrate actin-related protein and γ-tubulin. J. Cell Biol. *122*, 1301–1310.

Mitchison, T., and Kirschner, M.W. (1984). Dynamic instability of microtubule growth. Nature *312*, 237–242.

Mitchison, T., and Kirschner, M.W. (1986). Beyond self-assembly: from microtubules to morphogenesis. Cell *45*, 329–342.

Moreno, S., Klar, A., and Nurse, P. (1991). Molecular genetic analyses of fission yeast *Schizosaccharomyces pombe*. Methods Enzymol. *194*, 773–782.

Pierre, P., Scheel, J., Rickard, J.E., and Kreis, T.E. (1992). CLIP-170 links endocytotic vesicles to microtubules. Cell *70*, 887–900.

Radcliffe, P., Hirata, D., Childs, D., Vardy, L., and Toda, T. (1998). Identification of novel temperature-sensitive lethal alleles in essential β-tubulin and nonessential α2-tubulin genes as fission yeast polarity mutants. Mol. Biol. Cell *9*, 1757–1771.

Rickard, J.E., and Kreis, T.E. (1996). CLIPs for organelle-microtubule interactions. Trends Cell Biol. *6*, 178–183.

Riehemann, K., and Sorg, C. (1993). Sequence homologies between four cytoskeleton-associated proteins. Trends Biochem. Sci. *18*, 82–83.

Robinson, M.J., Stippec, S.A., Goldsmith, E., White, M.A., and Cobb, M.H. (1998). A constitutively active and nuclear form of the MAP kinase ERK2 is sufficient for neurite outgrowth and cell transformation. Curr. Biol. *8*, 1141–1150.

Rommelaere, H., Van Troys, M., Gao, Y., Melki, R., Cowan, N.J., Vandekerckhove, J., and Ampe, C. (1993). Eukaryotic cytoplasmic chaperonin contains t-complex polypeptide 1 and seven related subunits. Proc. Natl. Acad. Sci. USA *90*, 11975–11981.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor, NY: Cold Spring Harbor Laboratory

Stearns, T., Hoyt, M.A., and Botstein, D. (1990). Yeast mutants sensitive to antimicrotubule drugs define three genes that affect microtubule function. Genetics *124*, 251–262.

Tian, G., Huang, Y., Rommelaere, H., Vandekerckhove, J., Ampe, C., and Cowan, N.J. (1996). Pathway leading to correctly folded β-tubulin. Cell *86*, 287–296.

Tian, G., Lewis, S.A., Feierbach, B., Stearns, T., Rommelaere, H., Ampe, C., and Cowan, N.J. (1997). Tubulin subunits exist in an activated conformational state generated and maintained by protein cofactors. J. Cell Biol. *138*, 821–832.

Toda, T., Adachi, Y., Hiraoka, Y., and Yanagida, M. (1984). Identification of the pleiotropic cell cycle gene *NDA2* as one of two different α-tubulin genes in *Schizosaccharomyces pombe*. Cell *37*, 233–242.

Ursic, D., and Culbertson, M.R. (1991). The yeast homolog to mouse *Tcp-1* affects microtubule-mediated processes. Mol. Cell. Biol. *11*, 2629–2640.

Vainberg, I.E., Lewis, S.A., Rommelaere, H., Ampe, C., Vandekerckhove, J., Klein, H.L., and Cowan, N.J. (1998). Prefoldin, a chaperone that delivers unfolded proteins to cytosolic chaperonin. Cell *93*, 863–873.

Vega, L.R., Fleming, J., and Solomon, F. (1998). An α-tubulin mutant destabilizes the heterodimer: phenotypic consequences and interactions with tubulin-binding proteins. Mol. Biol. Cell *9*, 2349–2360.

Waterman-Storer, C.M., and Salmon, E.D. (1997). Microtubule dynamics: treadmilling comes around again. Curr. Biol. *7*, R360–R372.

Yaffe, M.B., Farr, G.W., Miklos, D., Horwich, A.L., Sternlicht, M.L., and Sternlicht, H. (1992). TCP1 complex is a molecular chaperone in tubulin biogenesis. Nature *358*, 245–248.

Yaffe, M.P., Hirata, D., Verde, F., Eddison, M., Toda, T., and Nurse, P. (1996). Microtubules mediate mitochondrial distribution in fission yeast. Proc. Natl. Acad. Sci. USA *93*, 11664–11668.

Yamashita, A., Watanabe, Y., and Yamamoto, M. (1997). Microtubule-associated coiled-coil protein Ssm4 is involved in the meiotic development in fission yeast. Genes Cells *2*, 155–166.