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ORIGINAL PAPER

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Characterisation of fission yeast *alp11* mutants defines three functional domains within tubulin-folding cofactor B

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Abstract The proper folding of tubulins prior to their incorporation into microtubules requires a group of conserved proteins called cofactors A to E. In fission yeast, homologues of these cofactors (at least B, D and E) are necessary for the biogenesis of microtubules and for cell viability. Here we show that the temperature-sensitive *alp11-924* mutant, which is defective in the cofactor B homologue, contains an opal nonsense mutation, which results in the production of a truncated $Alp11^{B}$ protein ($Alp11_{1–118}$). We isolated a $tRNA^{Trp}$ gene as a multicopy suppressor of this mutation, which rescues *alp11-924* by read-through of the nonsense codon. The truncated $Alp11_{1–118}$ protein lacks the C-terminal half of $Alp11^{B}$, consisting of a central coiled-coil region and the distal CLIP-170 domain found in a number of proteins involved in microtubule functions. Both of these domains are required for the maintenance of microtubule architecture in vivo. Detailed functional analyses lead us to propose that $Alp11^{B}$ comprises three functional domains: the N-terminal half executes the essential function, the central coiled-coil region is necessary for satisfactory maintenance of cellular α-tubulin levels, and the C-terminal CLIP-170 domain is required for efficient binding to α-tubulin.

Key words CLIP-170 · Cofactors · Microtubule · *Schizosaccharomyces pombe* · tRNA

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Introduction

Microtubules (MTs) play pivotal roles in a wide variety of cellular processes including chromosome separation, protein and mRNA transport, cell motility and morphogenesis. They are composed of polymers made up of α/β-tubulin heterodimers. The correct folding of tubulin requires members of a highly conserved subfamily of chaperones, known as CCT/TriC/c-cpn, which are GroEL- and Hsp60-related chaperonins (Gao et al. 1992; Melki et al. 1993; Rommelaere et al. 1993; Kubota et al. 1994). Furthermore, the formation of MTs is dependent on additional co-chaperonins, designated cofactors (Gao et al. 1993, 1994).

Cofactors were first identified biochemically in mammalian in vitro systems as protein factors needed for the production of assembly-competent α/β heterodimers. Subsequent protein purification and molecular cloning showed that cofactors comprise a group of five proteins designated cofactors A, B, C, D and E, (Llosa et al. 1996; Melki et al. 1996; Tian et al. 1996, 1997). Independent genetic experiments performed in yeasts have shown that these cofactors (except for cofactor C) are conserved through evolution. In budding yeast (*Saccharomyces cerevisiae*), the homologues of cofactors A (Rbl2p, Archer et al. 1995, 1998; Vega et al. 1998), B (Alf1p, Tian et al. 1997; Feierbach et al. 1999), D (Cin1p, Hoyt et al. 1990; Stearns et al. 1990) and E (Pac2p, Geiser et al. 1997; Hoyt et al. 1997) have been identified, and their involvement in MT-related function in vivo has been demonstrated.

In budding yeast the cofactor homologues are not essential for cell viability (Hoyt et al. 1990; Stearns et al. 1990; Archer et al. 1995; Tian et al. 1997), which appears to contradict the expectation based on the findings in mammalian systems, in which these cofactors are absolutely required for tubulin folding in vitro (reviewed in Lewis et al. 1997). In contrast, analyses in fission yeast (*Schizosaccharomyces pombe*) have shown clearly that the corresponding homologues, at least of cofactors B

(Alp11), D (Alp1) and E (Alp21) execute a function that is essential for cell viability (Hirata et al. 1998; Grishchuk and McIntosh 1999; Radcliffe et al. 1999). Furthermore a conserved small GTP-binding protein, Alp41 (Cin4p in budding yeast), is required for the cofactor homologue-dependent pathway (Radcliffe et al. 2000). Mutations, either deletion or conditional, in these homologues result in defective phenotypes very similar, if not identical, to those in tubulin genes, including abnormal mitosis, mislocalisation of mitochondria, nuclear displacement and growth polarity defects, and, most notably, failure to form normal MT structures (Umesono et al. 1983; Hiraoka et al. 1984; Toda et al. 1984; Ayscough et al. 1993; Yaffe et al. 1996; Mata and Nurse 1997; Radcliffe et al. 1998; Sawin and Nurse 1998). Based on data from fission yeast, we have proposed that there is hierarchy among the cofactor homologues in that they operate sequentially in a linear pathway, Alp11^{B}-Alp21^{E}/Alp41-Alp1^{D} (Radcliffe et al. 1999, 2000).

Alp11^{B} binds free α-tubulin molecules and overproduction results in lethality accompanied by the disappearance of MTs from the cell. This toxicity of Alp11^{B} is most probably ascribable to the absorption of α-tubulin, as simultaneous overproduction of α-tubulin rescues lethality (Radcliffe et al. 1999). Alp11^{B} contains, at its C-terminus, the conserved "CLIP-170 domain" which consists of 50 amino acid residues rich in glycine and is found in a number of proteins involved in MT-dependent processes (reviewed in Rickard and Kreis 1996). These include CLIP-170/Restin, p150^{Glued}, budding yeast Bik1p and cofactors B and E (although fission yeast Alp21^{E} does not contain this domain). Our previous experiments have shown that the CLIP-170 domain of Alp11^{B} is required for efficient binding of Alp11^{B} to free α-tubulin molecules. In agreement with this, a truncated Alp11^{B} protein which lacks this CLIP-170 domain is no longer toxic upon overproduction. Unexpectedly, however, this truncated protein still appears to execute its function in vivo as efficiently as the intact Alp11^{B} protein (Radcliffe et al. 1999).

In the present study we report that the temperature-sensitive (ts) *alp11-924* allele contains an opal nonsense mutation, which is suppressible by tRNA^{Trp}. This finding provoked further investigation of the importance of a predicted coiled-coil region adjacent to the CLIP-170 domain. Our results show that the coiled-coil region proximal to the CLIP-170 domain is crucial for Alp11^{B} protein stability and for the maintenance of α-tubulin levels in vivo.

Materials and methods

Strains, media, genetic techniques and nomenclature

Strains used in this study are listed in Table 1. Complete medium, YPD (1% yeast extract, 2% polypeptone, 2% dextrose), YE5S, modified synthetic EMM2 and MEA4S (Moreno et al. 1991) have all been described. Standard procedures for *S. pombe* genetics were followed according to Moreno et al. (1991). Proteins are designated by an uppercase initial letter, e.g. Alp11.

Table 1 Strains used

Strain	Genotype	Source/reference
HM123	*h⁻ leu1*	Laboratory stock
DH924	*h⁻ leu1 alp11-924*	Radcliffe et al. (1999)
PR14	*h⁻ leu1 ura4 his7 ade6 alp11$_{1–163}$::ura4⁺*	Radcliffe et al. (1999)
PR19	*h⁻/h⁺ leu1/leu1 ura4/ura4 his7/his7 ade6-M210/ade6-M216 alp11$_{1–118}$::ura4⁺/+*	This study
PR20	*h⁻ leu1 ura4 his7 ade6 alp11$_{1–118}$::ura4⁺*	This study

Cloning of the tRNA^{Trp}-encoding *sap11⁺* gene as a multicopy suppressor of *alp11-924*

An *S. pombe* genomic library constructed in the vector pDB248 was used for the isolation of genes which complemented the ts *alp11-924* mutant (DH924, Table 1). Transformants were incubated for 6 days at 26 °C, replica-plated onto YPD plates and incubated at 36 °C. Out of a total of 6500 colonies screened, 10 were capable of growing at 36 °C and segregation analysis showed that the Ts⁺ phenotype was plasmid dependent. Plasmid DNAs were recovered from *E. coli*, and restriction mapping indicated that they contained overlapping inserts (two different plasmids) and did not correspond to either *alp11⁺* or *alp21⁺* (the gene was designated *sap11⁺*).

Nucleic acid preparation and manipulation

Standard molecular biological techniques were followed as described by Sambrook et al. (1989). Enzymes were used as recommended by the suppliers (New England Biolabs, Beverly, Mass.). Nucleotide sequence was determined by the dideoxy method (Sanger et al. 1977).

Construction of chromosomal truncation of *alp11*

Oligonucleotides 100 bases long were designed to create a truncation mutant of *alp11* by using a PCR-generated fragment containing the *ura4⁺* marker (Bähler et al. 1998), in which a pair of stop codons (TAAGCTTAG, stop codons are underlined) were introduced at the position of Trp^{119} in one copy of the *alp11⁺* gene in a diploid strain (PR19, Table 1). Tetrad dissection yielded two normal-sized colonies and two small colonies. The small colonies were Ura⁺, indicating that the C-terminal truncation is partially functional. The Ura⁺ colonies were ts and cold-sensitive (cs) for growth (strain PR20).

Cloning of the ts *alp11-924* gene and determination of the mutation site

To determine the site of the mutation in the ts *alp11-924* allele, the entire ORF was cloned from the ts *alp11-924* mutant into pUC19 by PCR. Sequencing of two clones derived from independent PCRs showed, in both cases, a single substitution, from G to A, at position 357, which corresponded to the replacement of the codon for Trp^{119} (TGG) by the opal termination codon (TGA, the A of initiator methionine is defined as +1 and the mutated base is underlined).

Immunochemical assays

Fission yeast whole-cell extracts were prepared, using glass beads to disrupt cells as described previously (Vega et al. 1998; Radcliffe et al. 1999). 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), rat monoclonal anti-mouse TCP-1 antibody (provided by Dr. Keith Willison) and monoclonal anti-Cdc2 (PSTAIR) antibody (provided by Dr. Masakane Yamashita) were also used. Horseradish peroxidase-conjugated goat anti-rabbit IgG, goat anti-mouse IgG (Bio-Rad Laboratories Ltd., Hercules, Calif.) and a chemiluminescence system (ECL, Amersham, Little Chalfont, Bucks., UK) were used to detect bound antibody.

Indirect immunofluorescence microscopy

Cells were fixed with methanol at −80 °C and primary antibodies (TAT-1) were applied, followed by Cy3-conjugated sheep anti-mouse IgG (Sigma Chemical Co.). Image processing was performed using a chilled video-rated CCD camera (Model C5985; Hamamatsu) with Hamamatsu software and Adobe Photoshop (version 4).

Sucrose gradient centrifugation

Aliquots (2 mg, 100 µl) of yeast cell extract prepared in HB buffer were loaded on the top of linear sucrose gradients (4.5 ml, 15%–40%), and centrifuged at 40,000 rpm for 12 h at 4 °C.

Results

The *alp11-924* mutation is suppressed by a multicopy plasmid containing a $tRNA^{Trp}$ gene

In order to identify additional factors that potentially interact with the tubulin-folding pathway, an *S. pombe* genomic library was used to further screen for genes which complemented the ts *alp11-924* mutation, which is defective in the cofactor B homologue. In addition to plasmids which contained $alp11^+$ or the gene for the cofactor E homologue, $alp21^+$, as reported previously (Radcliffe et al. 1999), two novel plasmids containing overlapping inserts were isolated. Subsequent subcloning analysis indicated that the complementing activity resided in a short insert of only 285 bp (Fig. 1A, B). Analysis of the nucleotide sequence of this fragment did not reveal any obvious ORFs; however, it did contain a $tRNA^{Trp}$ gene ($sap11^+$) in which the anticodon is 3′ACC5′ (Fig. 1C).

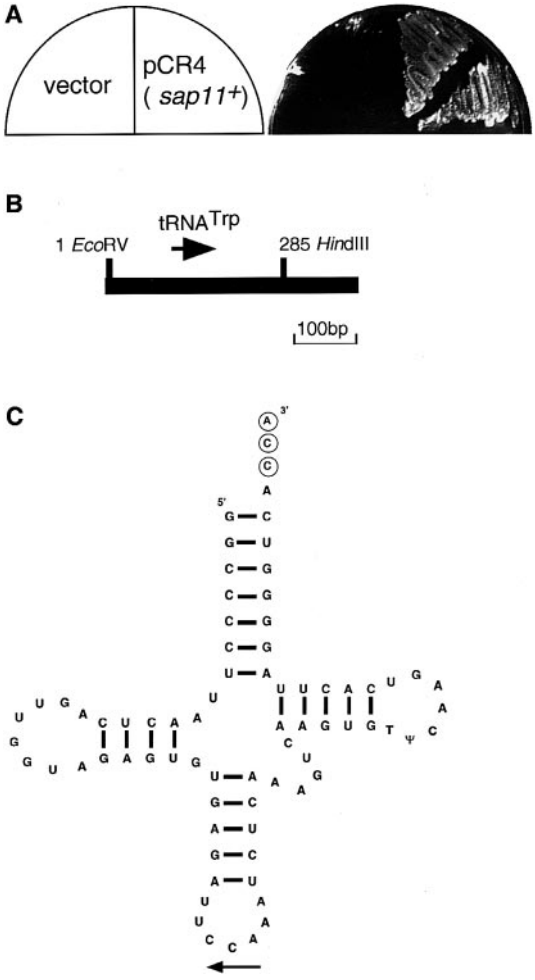

Fig. 1A–C Isolation of $tRNA^{Trp}$ as a multicopy suppressor of *alp11-924* **A** Suppression of ts *alp11-924* by a plasmid containing $tRNA^{Trp}$ ($sap11^+$). *alp11-924* cells bearing a vector plasmid (*left*) or plasmid containing $sap11^+$ (pCR4, *right*) were streaked on rich medium and incubated for 3 days at 36 °C. **B** Restriction map of the insert DNA containing $sap11^+$. The *Eco*RV site (*left*) is denoted as +1. The $tRNA^{Trp}$ gene is indicated by the *arrow*. **C** Structure of the $tRNA^{Trp}$ encoded by $sap11^+$. The predicted secondary structure of $tRNA^{Trp}$ is shown with anticodon (3′ACC5′, *arrow*) and base pairing (*bars*). The terminal CCA is added (*circles*). The nucleotide sequence data reported in this paper are available in the DDBJ/EMBL/Genbank nucleotide sequence databases under the Accession No. AB019620 ($sap11^+$)

alp11-924 is an opal mutation

$tRNA^{Trp}$ is known to be capable of reading through the opal terminator codon (UGA) even under normal conditions, although at a low frequency (Watson et al. 1987). Bearing this in mind, we isolated genomic DNA from an *alp11-924* strain and sequenced the ts *alp11-924* mutant allele. As shown in Fig. 2A, we found a single point mutation (G → A) at 357 (the A of the initiator methionine is defined as +1, Fig. 2A). In wild type, G^{357} corresponds to the third nucleotide of the codon for Trp^{119} (TG<u>G</u>, position 357 is underlined). However, in the *alp11-924* mutant, A^{357} results in an opal stop codon (TG<u>A</u>), thereby causing premature termination of the $Alp11^B$ protein (Fig. 2B). As expected, suppression of the *alp11* mutant by $sap11^+$ was specific to the *alp11-924* allele; the tRNA gene was incapable of rescuing the *alp11*-deletion strain (data not shown).

In the *alp11-924* mutant a truncated $Alp11^B$ protein is reverted to wild-type length by suppressor $tRNA^{Trp}$

To confirm that a truncated $Alp11^B$ protein was being produced in the *alp11-924* mutant as expected, immunoblotting analysis was performed using anti-Alp11 antibody. It was found that the *alp11-924* mutant ap-

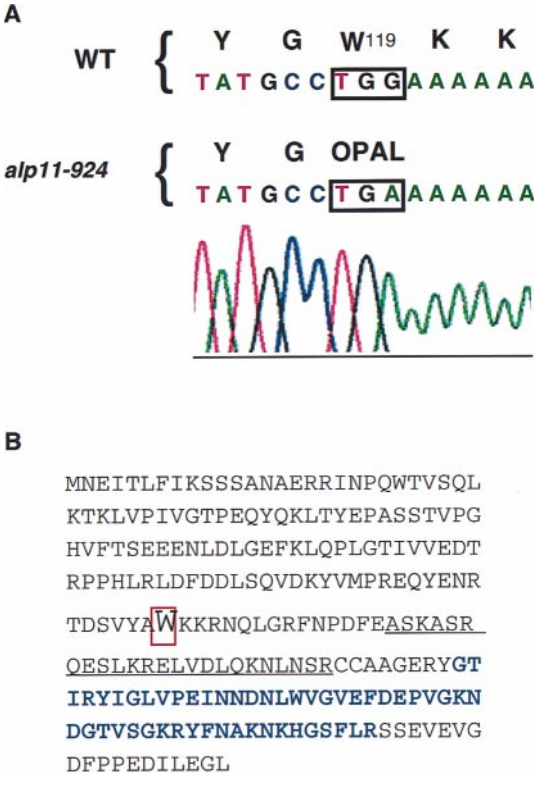

Fig. 2A, B *alp11-924* is an opal mutation. **A** Nucleotide sequence of *alp11-924*. The nucleotide sequence of a part of the wild type (WT) and *alp11-924* alleles of the *alp11* gene are shown, together with the predicted amino acid sequence (one-letter code). Raw sequencing data are also shown for *alp11-924*, in which G^{357} is mutated to A^{357}. The Trp^{119} codon (TGG, WT) and the opal termination codon (TGA, *alp11-924*) are *boxed*. **B** The predicted amino acid sequence of $Alp11^{B}$. Trp^{119} is *enlarged and boxed*. The CLIP-170 domain is shown in *bold blue type*, whilst the proximal coiled-coil region is *underlined* (see Fig. 5C)

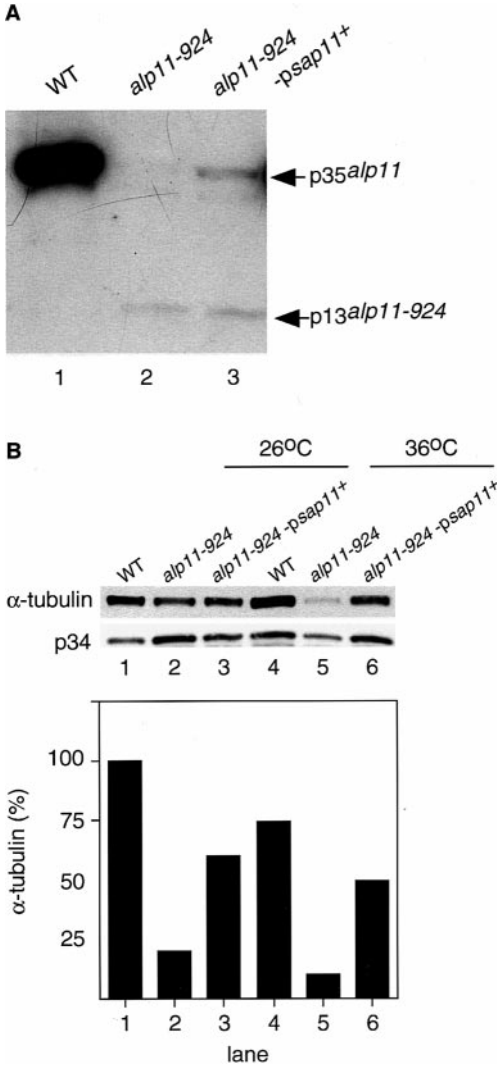

Fig. 3A, B Level of the $Alp11^{B}$ and α-tubulin proteins in the *alp11-924* mutant. **A** Truncation of the $Alp11^{B}$ protein. Wild-type cells (lane 1), *alp11-924* (lane 2) or *alp11-924* cells containing plasmids carrying $sap11^{+}$ (lane 3) were grown in minimal medium at 26 °C. Cell extracts were prepared and immunoblotting performed with anti-Alp11 antibody. Wild-type $Alp11^{B}$ (p35) and the truncated mutant protein (p13) are indicated by the *arrows*. **B** Level of α-tubulin. The strains used in **A** were grown in minimal medium at 26 °C (lanes 1–3), shifted to 36 °C and incubated for a further 8 h (lanes 4–6). Western analysis was performed with anti-α-tubulin or anti-Cdc2 (p34) antibodies. The histogram shows the level of α-tubulin relative to that of Cdc2, as estimated by densitometry of the immunoblots

parently lacked wild-type $p35^{alp11}$; instead, a much fainter band of around 13 kDa was observed (Fig. 3A, lane 2). This size is consistent with that predicted if truncation were occurring at amino acid position 118 (the calculated molecular weight is 13.5 kDa). In the *alp11-924* mutant transformed with multicopy plasmids carrying $tRNA^{Trp}$-encoding $sap11^{+}$, a small but clearly significant amount of wild-type $p35^{alp11}$ was produced (lane 3). This result confirms the finding that *alp11-924* is an opal mutation which is suppressed by $tRNA^{Trp}$-mediated read-through of the stop codon.

Our previous results indicated that in an *alp11-924* mutant, endogenous α-tubulin levels are decreased (Radcliffe et al. 1999). Given the appearance of full-length $p35^{alp11}$ as a result of suppression by the multicopy $tRNA^{Trp}$-encoding gene $sap11^{+}$, this defect in α-tubulin levels should also be complemented. As shown in Fig. 3B, that was indeed the case. In *alp11-924* mutant cells containing $sap11^{+}$ the level of α-tubulin was enhanced to a significant extent at the restrictive temperature (36 °C, lanes 5 and 6) although not to wild-type levels (compare lanes 4 and 6). Therefore, a high dosage of $tRNA^{Trp}$ results in read-through of the opal mutation, which leads to the appearance of full-length $Alp11^{B}$ ($p35^{alp11}$) and thus normal α-tubulin levels.

Two mechanisms underlie the temperature sensitivity of *alp11-924*

We wished to discover the reason why *alp11-924* cells are ts, and in particular to determine how this mutant survives at the permissive temperature in spite of the apparent lack of intact $p35^{alp11}$. Two explanations can be

envisaged. One possibility is that the truncated protein itself is ts, i.e. p13 is functional at 26 °C, but not at 36 °C. The other is that the viability of *alp11-924* cells at 26 °C is dependent upon a small amount of $p35^{alp11}$ being translated by endogenous $tRNA^{Trp}$-mediated read-through of the opal codon, but that the quantity of $p35^{alp11}$ thus produced (which must be below the detection level of our immunoblotting experiments, Fig. 3A), is for some reason insufficient to sustain growth at higher temperatures. To distinguish between these two possibilities, a heterozygous diploid strain was constructed in which double stop codons were introduced into one copy of the $alp11^+$ gene at the position corresponding to Trp^{119} of $Alp11^B$ (see Materials and methods). The double stop codons (TAAGCTTAG, stop codons are underlined) ensure truncation of the $Alp11^B$ protein without allowing any potential read-through by chromosomal $sap11^+$ (the product derived from this truncated *alp11* was designated $Alp11_{1–118}$). Thus, if the former possibility were true, the resulting haploid strain, containing $Alp11_{1–118}$ as sole source of the cofactor B homologue, would show phenotypes identical to those of *alp11-924*; i.e. viable, ts and with modest defects at the permissive temperature (26 °C). On the other hand, if the latter notion were true, haploid cells containing $Alp11_{1–118}$ would be inviable, as are *alp11* disruptants (Radcliffe et al. 1999).

Tetrad dissection of a heterozygous diploid showed that a haploid strain containing $Alp11_{1–118}$ was viable, although mutant cells were more severely retarded for growth than *alp11-924*; they were ts (Fig. 4A) and also cs (18 °C, not shown), and even at the permissive temperature (26 °C), showed markedly slow growth (Fig. 4A) with altered cell shape (Fig. 4B, upper left). The morphology of cells containing $Alp11_{1–118}$ became more abnormal when the strain was incubated at 36 °C (Fig. 4B, upper right). For comparison, the cell morphology of a ts *alp11-924* strain grown at 26 °C and 36 °C is also shown (Fig. 4B, middle panel). This result suggested that the two possibilities described above are in fact both operative. The *alp11-924* cells must benefit from read-through of the opal codon by chromosomal $sap11^+$, as cells containing $Alp11_{1–118}$ were more impaired for growth at 26 °C than *alp11-924* cells. Nevertheless, $Alp11_{1–118}$ must be functional at 26 °C, since cells containing this truncated protein are viable, and can grow and divide at this temperature.

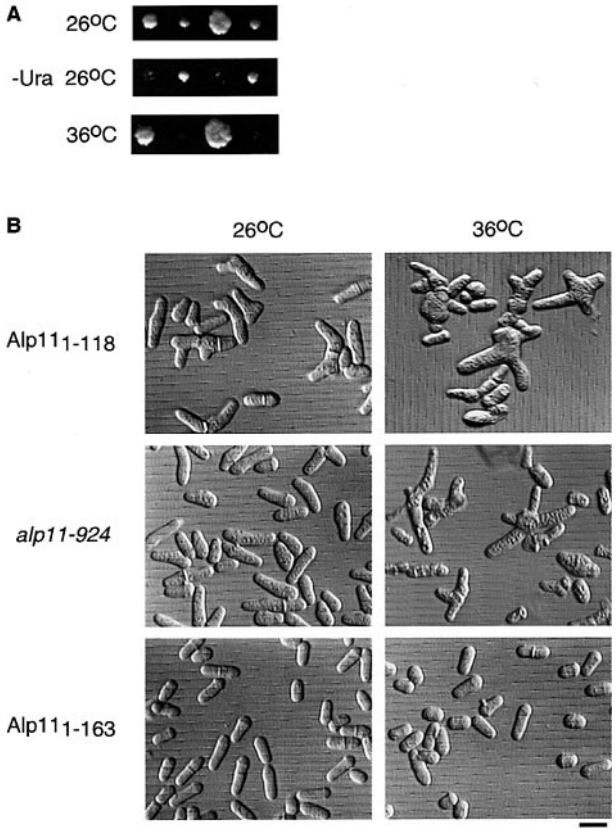

Fig. 4A, B Effects of C-terminal truncation of the Alp11 protein. **A** Construction of haploid cells containing $Alp11_{1–118}$. A tetrad derived from a heterozygous diploid (PR19) in which one chromosomal copy of $alp11^+$ has been modified to encode the truncated $Alp11^B$ protein ($Alp11_{1–118}$) is shown. Cells were grown rich medium at 26 °C or 36 °C for 3 days (bottom), or on minimal medium lacking uracil (Ura) at 26 °C for 4 days. **B** Cell morphology of strains containing truncated $Alp11^B$ proteins. Haploid cells containing $Alp11_{1–118}$ (PR20, *top*) or $Alp11_{1–163}$ (PR14, *bottom*) or *alp11-924* mutant cells (DH924, *middle*) were streaked on rich medium and incubated for 2 days either at 26 °C (left) or at 36 °C (right). The *bar* indicates 10 μm

A coiled-coil region proximal to the CLIP-170 domain of $Alp11^B$ is required for the maintenance of α-tubulin levels

To examine the level of tubulin molecules in $Alp11^B$ truncation mutants, immunoblotting with anti-α- and anti-β-tubulin antibodies was performed. The mutants used were $Alp11_{1–163}$, which lacks only the C-terminal CLIP-170 domain (Radcliffe et al. 1999), and $Alp11_{1–118}$. Anti-Alp11 antibody was used to detect the truncated $Alp11^B$ protein in each strain. As shown in Fig. 5A (lower panel), mutant cells containing truncated *alp11* genes produced p25 or p13, instead of p35, as expected. It was also found that the amount of α-tubulin was substantially reduced in cells containing $Alp11_{1–118}$ (Fig. 5A, lane 2, upper panel). However, cells containing the less severe truncation of $Alp11^B$ ($Alp11_{1–163}$) showed normal growth rate and morphology and were as resistant to the anti-microtubular drug thiabendazole as wild-type cells (Figs. 4B and 5B; note that the *alp11-924* strain is hypersensitive to this drug) and also contained a normal amount of α-tubulin (Fig. 5A, lane 3, upper panel). In contrast to the findings for α-tubulin, the level of β-tubulin showed no significant change from the wild-type level in either of these mutants (Fig. 5A, centre panel).

The drastic phenotypic difference between $Alp11_{1–118}$ (severely retarded) and $Alp11_{1–163}$ (normal) prompted us to examine the structural features, if any, of the region

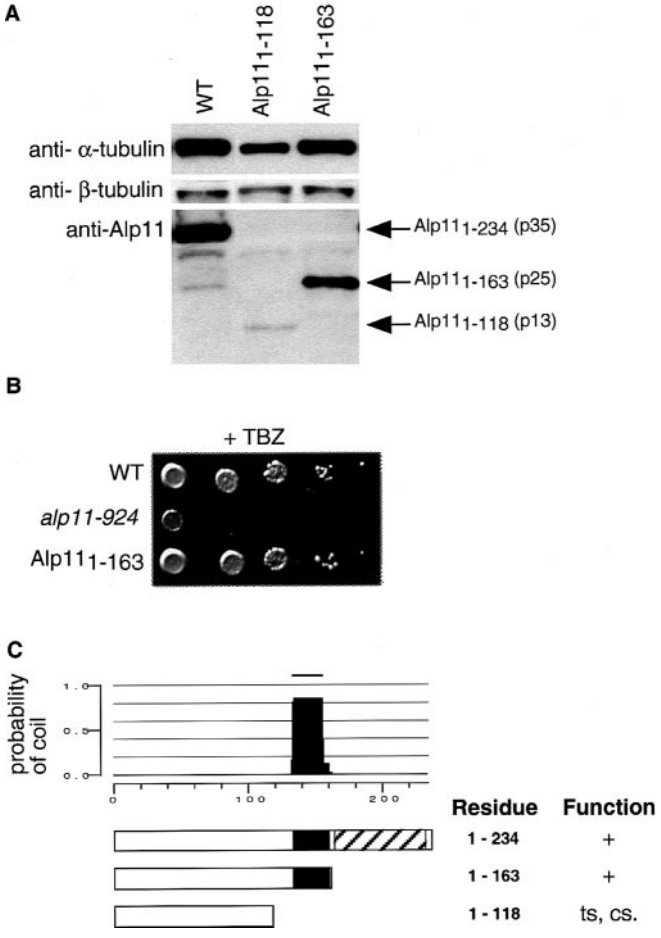

Fig. 5A–C A truncation derivative of Alp11^B that lacks both the coiled-coil and CLIP-170 domains results in a reduction in α-tubulin levels. **A** Reduced levels of α-tubulin in cells containing Alp11$_{1–118}$. Western analysis with anti-α-tubulin (*top*), anti-β-tubulin (*middle*) or anti-Alp11 (*bottom*) antibody was performed using cell extracts prepared from wild type cells (HM123, lane 1), and cells containing Alp11$_{1–118}$ (PR20, lane 2) or Alp11$_{1–163}$ (PR14, lane 3). The positions of each Alp11^B protein (wild-type p35, Alp11$_{1–163}$ p25 and Alp11$_{1–118}$ p13) are shown by the *arrows*. **B** Cells containing Alp11$_{1–163}$ show normal levels of resistance to thiabendazole. Serial dilutions of wild-type cells (HM123), *alp11-924* mutant cells (DH924) or cells containing Alp11$_{1–163}$ (PR14) were spotted onto plates of rich medium containing thiabendazole (TBZ, 20 μg/ml); The 10^5 cells per spot in the left column were diluted 10-fold in subsequent rows) and incubated at 29 °C for 3 days. **C** Predicted coiled-coil structure and functional profile of the truncated Alp11^B proteins. Coiled-coil probability was determined with the Macstripe 2.0 program using window lengths of 14 and 21 amino acid residues. The coiled-coil region and the CLIP-170 domain are shown by *filled* and *hatched boxes*, respectively

proximal to the CLIP-170 domain. It was found that the region between amino acids 119 and 163 contains a coiled-coil structure (Fig. 5C). Thus it was concluded that the coiled-coil region, but not the distal CLIP-170 domain, of Alp11^B is essential for the maintenance of steady state levels of free α-tubulin molecules. Also the data shows that Alp11^B specifically regulates α-tubulin, which is in line with biochemical work performed in higher eukaryotes (Tian et al. 1997).

Microtubules are defective in cells containing truncated Alp11^B proteins

Next we sought to examine MT structures in *alp11* truncation mutants. As shown in Fig. 6A and B, MT structures were abnormal in cells containing Alp11$_{1–118}$; MTs were fewer and shorter or seen as discrete dots. These abnormal MTs are reminiscent of those in cells treated with anti-microtubule drugs (Fig. 6C).

Furthermore, even cells containing Alp11$_{1–163}$ also showed incomplete MTs, although defects appeared less severe than in cells expressing Alp11$_{1–118}$; filamentous structures were still observed, although shorter in length (Fig. 6D). This result showed that, in the absence of the CLIP-170 domain and/or coiled-coil region, Alp11^B function becomes significantly compromised; MTs appeared more unstable.

Interaction between chaperonins and Alp11^B or tubulin is transient in vivo

Next we investigated whether chaperonins (Lewis et al. 1992; Yaffe et al. 1992) form a stable complex with Alp11^B or tubulins in vivo. Total cell extracts of fission yeast were fractionated by sucrose gradient centrifugation, and immunoblotting was performed with anti-TCP-1 (provided by Dr. Keith Willison), anti-Alp11^B or anti-tubulin antibody. As shown in Fig. 7, the majority of the chaperonin proteins were detected in fractions corresponding to large complexes (>20S, fractions 9–11). In contrast, Alp11^B and tubulins were found much further up the gradient (fractions 1–5 for Alp11^B and 3–5 for tubulins). Immunoprecipitation analysis under conditions in which Alp11^B and α-tubulin interacted (Radcliffe et al. 1999) failed to show co-precipitation of chaperonins and Alp11^B (data not shown). These results indicate that the chaperonin complex and Alp11^B or tubulins do not form a stable complex in the cell and, therefore, that physical interaction must be weak or transient in vivo.

Discussion

In the current study we have shown that the fission yeast cofactor B homologue, Alp11^B, is composed of three functional domains: the N-terminal half, the central coiled-coil region and the C-terminal CLIP-170 domain.

The role of the coiled-coil region of Alp11^B

Our results suggest that the coiled-coil region is required for the intrinsic stability of Alp11^B, as the level of protein is greatly reduced in its absence. Furthermore it is clear that a mutant form of Alp11^B lacking the coiled-coil region, in addition to the CLIP-170 domain, fails to maintain normal cellular α-tubulin levels. This could

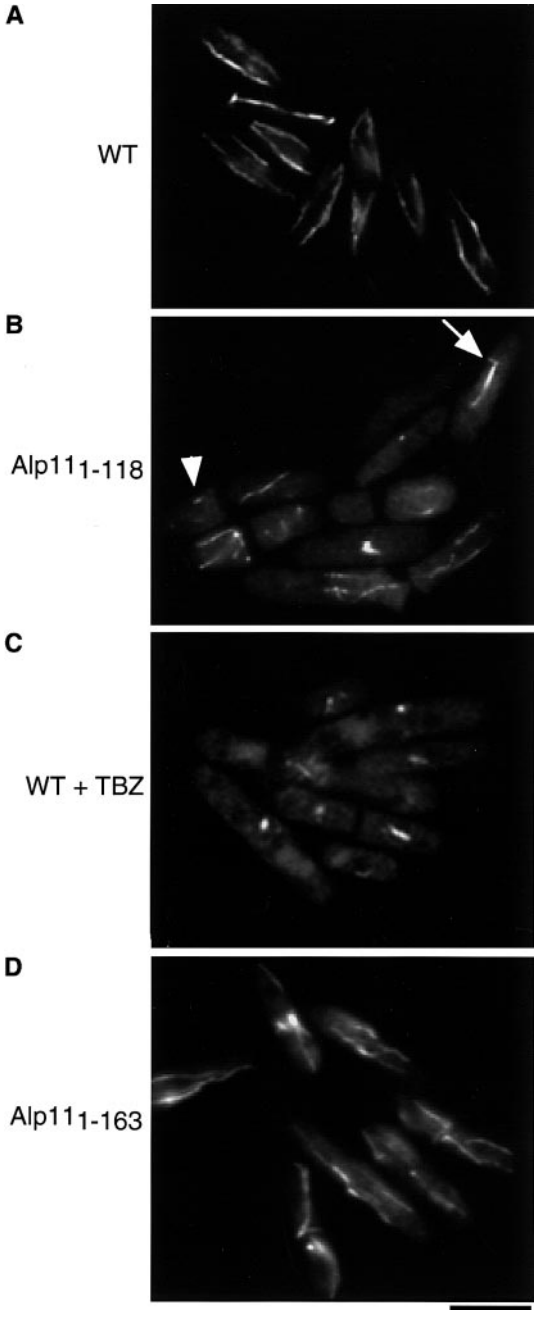

Fig. 6A–D Defective microtubule structures in cells containing the truncated Alp11 proteins. Wild type cells (HM123, **A** and **C**), cells containing $Alp11_{1–118}$ (PR20, **B**) or $Alp11_{1–163}$ (PR14, **D**) were grown in rich medium at 30 °C. Cells shown in **C** are wild type cells carrying plasmids containing *alp21*$^+$ (pREP-*alp21*$^+$), and were incubated for 2 h in medium containing thiabendazole (50 μg/ml). Cells were fixed with methanol and processed for immunofluorescence microscopy using anti-α-tubulin antibody (TAT-1). $Alp11_{1–118}$ cells which contain shorter and misoriented or dotted microtubule structures are indicated by the *arrowhead* and *arrow*, respectively. The *bar* indicates 10 μm

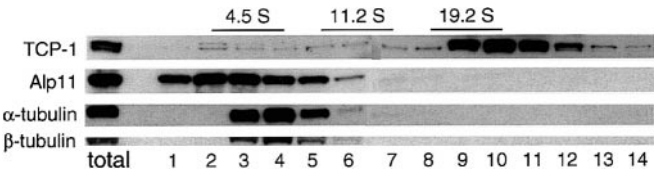

Fig. 7 Size fractionation of Alp11^B, tubulin and chaperonins. Sucrose-gradient centrifugation. Cell extracts prepared from wild-type cells were centrifuged through a 15–40% sucrose gradient, and fractions were analysed by immunoblotting with anti-TCP-1, anti-Alp11, anti-α-tubulin and anti-β-tubulin antibodies, as indicated. Positions of sedimentation markers (19.2S, thyroglobulin; 11.2S, catalase; 4.5S, BSA) and fraction numbers are also shown. Aliquots (25 μg) of the total cell extracts used for centrifugation were run in the lane on the far left (total)

simply be a direct consequence of an inadequate quantity of Alp11^B. An alternative possibility is that this region has (a) specific role(s), for example, α-tubulin-binding, in conjunction with the distal CLIP-170 domain. This would explain why $Alp11_{1–163}$ retains some binding capability, although reduced, for α-tubulin (Radcliffe et al. 1999). As cells appear able to grow normally with a greatly reduced amount of p35^{alp11} (see below), we think it likely that the coiled-coil region does make an important contribution to the function of Alp11^B, in addition to stabilising the protein. $Alp11_{1–163}$ is capable of executing its role in ensuring cell viability as efficiently as wild-type p35^{alp11}. The stable binding between Alp11^B and α-tubulin is therefore not essential. Nonetheless, the CLIP-170 domain is important for maintaining MT structures, and without this domain, MTs become highly unstable. Vertebrate cofactor B, although not the budding yeast homologue (Alf1p), contains an analogous coiled-coil region adjacent to the CLIP-170 domain (Tian et al. 1997). Moreover, it appears that many proteins that contain the CLIP-170 domain possess regions of coiled-coils as well, although their lengths and relative positions vary (proximal or distal to the CLIP-170 domain, Rickard and Kreis 1996). Co-existence of the CLIP-170 domain and coiled-coils may be a common feature of this group of proteins.

Minimal quantity of Alp11^B required for microtubule biogenesis and cell viability

An *alp11-924* mutant containing multicopy plasmids bearing the tRNA^{Trp}-encoding gene *sap11*$^+$ is capable of growing and dividing at the restrictive temperature. Despite this apparent phenotypic suppression, very little p35^{alp11} is produced by read-through in these cells (at most one-tenth of that in wild type). Also the growth properties of *alp11-924* and the truncation mutant ($Alp11_{1–118}$) are very different at 26 °C: the *alp11-924* strain appears to grow as fast as wild type with relatively normal morphology, whilst growth of the $Alp11_{1–118}$ strain is greatly retarded, with altered growth polarity. Based on these differences, we assume that, although undetectable by immunoblot analysis, *alp11-924* strains must contain some p35^{alp11} as a result of read-through by the chromosomal *sap11*$^+$ gene. It appears, therefore, that this small amount of Alp11^B is sufficient to support normal growth. In wild-type cells Alp11^B is a relatively

abundant protein (levels are 10-fold higher than those of Alp21^{E}, Radcliffe et al. 1999). This high level of Alp11^{B} may not be essential for the survival of the cell, as its crucial role may be to capture α-tubulin from the chaperonin complex – a function which could require only a small number of Alp11^{B} molecules.

The essential requirement for Alp11^{B} can be bypassed under a number of conditions. These include augmented levels of Alp21^{E} or Alp1^{D} or spontaneous mutations which arise during the germination of spores deleted for the *alp11*$^{+}$ gene (Hirata et al. 1998; Radcliffe et al. 1999, 2000). The requirement for Alp11^{B} function is, thus, less stringent than that for Alp21^{E} and Alp1^{D} in fission yeast. This is in agreement with findings from in vitro biochemical experiments (Tian et al. 1997).

Suppression of the UGA opal mutation by tRNA^{Trp}

The isolation of the tRNA^{Trp}-encoding *sap11*$^{+}$ gene as a multicopy suppressor of the ts *alp11-924* mutant led us to investigate the mechanisms underlying this suppression. *alp11-924* was subsequently identified as an opal mutation; multicopy *sap11*$^{+}$ permits read-through translation of the codon, thereby producing full-length functional p35^{alp11}. As far as we know, this is the first in vivo demonstration in fission yeast that tRNA^{Trp} is capable of reading through the UGA termination codon. In budding yeast, a similar suppression is observed in *leu2-2* and *his4-260* TGA nonsense mutations (Ong et al. 1997). In addition many plant RNA viruses take advantage of tRNA^{Trp}-mediated-read-through in order to express some ORFs (Urban et al. 1996).

We have investigated termination codon usage in fission yeast ORFs to examine which, if any, is used preferentially. Among 40 randomly selected ORFs, the distribution of the termination codons is 8 UGA, 23 UAA and 9 UAG. There appears to be a preference for UAA, however, specific avoidance of UGA to prevent undesirable read-through does not seem to occur in the fission yeast genome. The fact that read-through of the UGA stop codon by tRNA^{Trp} has been tolerated, rather than selected against, suggests that in some circumstances this may be beneficial and could be of interest for further study from an evolutionary point of view.

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Note added in proof. We have recently shown that, unlike $Alp11^{B}$, $Alp21^{E}$ and $Alp1^{D}$, the fission yeast cofactor A homologue, Alp31, is non-essential for cell viability, despite $Alp31^{A}$ playing an important role in microtubule biogenesis in parallel with the above three proteins [Radcliffe. PA., Garcia, MA. and Toda, T. (2000) Genetics in press. ''The cofactor-dependent pathways for α- and β-tubulins in microtubule biogenesis are functionally different in fission yeast''].

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