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Essential role of tubulin-folding cofactor D in microtubule assembly and its association with microtubules in fission yeast

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The main structural components of microtubules are α- and β-tubulins. A group of proteins called cofactors are crucial in the formation of assembly-competent tubulin molecules *in vitro*. Whilst an *in vitro* role is emerging for these cofactors, their biological functions *in vivo* remain to be established. In order to understand the fundamental mechanisms that determine cell polarity, we have screened for fission yeast mutants with altered polarity. Here we show that *alp1*$^+$ encodes a homologue of cofactor D and executes a function essential for cell viability. A temperature-sensitive *alp1* mutant shows a variety of defects including abnormal mitoses, loss of microtubule structures, displacement of the nucleus, altered growth polarity and asymmetrical cell division. Overexpression of Alp1 is lethal in wild-type cells, resulting in altered cell shape, but is rescued by co-overexpression of β-tubulin. Alp1 co-localizes with microtubules, both interphase arrays and mitotic spindles. Furthermore, Alp1 binds to and co-sediments with taxol (paclitaxel)-stabilized porcine microtubules. Our results suggest that, in addition to a function in the folding of β-tubulin, cofactor D may play a vital role in microtubule-dependent processes as a microtubule-associated protein.

Keywords: cofactor D/fission yeast/MAPs/microtubules/polarity

Introduction

Microtubules constitute ubiquitous cytoskeletal elements and are important for a wide variety of cellular processes. They assemble from heterodimers consisting of α- and β-tubulins. It is generally believed that proteins other than tubulins are responsible for conferring the dynamic nature of microtubules in the living cell and for the correct folding and assembly of tubulin molecules (Mitchison and Kirschner, 1984, 1986; Hirokawa, 1994; Mandelkow and Mandelkow, 1995). These regulatory proteins include microtubule-associated proteins (MAPs), γ-tubulin, motor proteins and molecular chaperones (Lee, 1993; Hyman and Karsenti, 1996; Liang and MacRae, 1997). To understand the molecular basis for microtubule-dependent processes such as cell polarity, chromosome segregation and motility, it is essential to identify these regulators and elucidate their functions in microtubule dynamism in the cell.

Although classical experiments have established the idea that all the information to form the correct tertiary structure of a protein should exist in its primary amino acid sequence (Anfinsen, 1973), the proper folding of many proteins has been shown to be assisted by a set of proteins, known as molecular chaperones. Molecular chaperones exist ubiquitously in all organisms and constitute several large protein families (Rassow *et al.*, 1997). Originally, it was thought that chaperones aid the correct folding of other proteins only under conditions of extreme stress such as heat shock, but it is now clear that these proteins execute their roles in normal growth conditions (for review, see Bukau *et al.*, 1996; Hartl, 1996; Johnson and Craig, 1997). They participate in diverse processes such as subcellular compartmentalization of target proteins and disposal of proteins by degradation (Gottesman *et al.*, 1997).

In the case of the assembly of components of the eukaryotic cytoskeleton such as actin-based microfilaments and tubulin-based microtubules, CCT/TriC/c-cpn, a eukaryotic member of GroEL and Hsp60 families (called chaperonins), is required for the correct folding of actin and tubulin molecules, which facilitates their assembly into filamentous structures (Frydman *et al.*, 1992; Gao *et al.*, 1992; Yaffe *et al.*, 1992; Melki *et al.*, 1993; Sternlicht *et al.*, 1993). Interestingly, whilst CCT/TriC/c-cpn is the sole requirement for the folding of actin and γ-tubulin, in the case of α- and β-tubulin, a group of proteins, called cofactors, consisting of at least five polypeptides (A, B, C, D and E; Gao *et al.*, 1994; Tian, *et al.*, 1996, 1997) are required for the production of correctly folded and assembly-competent tubulin molecules (Gao *et al.*, 1992, 1993; Rommelaere *et al.*, 1993; Tian *et al.*, 1995, 1996, 1997). Although extensive biochemical work has been performed to address the roles of these cofactors in the folding and assembly of tubulins *in vitro*, insight into their *in vivo* functions remains limited. It has only recently become clear, based on mainly genetic analyses performed in budding yeast, that these cofactors in concert execute important roles in tubulin-dependent processes (Archer *et al.*, 1995; Hoyt *et al.*, 1997; Tian *et al.*, 1997).

Fission yeast is an ideal system for the study of the cytoskeleton and polarity control. The cell is rod-shaped

with uniform diameter, and growth polarity is strictly regulated in a cell cycle-dependent manner; growth occurs only in the tips in either a mono- or bidirectional manner, depending on cell cycle stage (Mitchison and Nurse, 1985). Microtubules show dynamic behaviour in the cell cycle; the interphase cytoplasmic array is replaced by mitotic nuclear spindles upon the onset of M phase (Marks *et al.*, 1986; Hagan and Hyams, 1988). Two α-tubulin (*nda2*$^+$ and *atb2*$^+$, Toda *et al.*, 1984; Adachi *et al.*, 1986), one β-tubulin (*nda3*$^+$, Hiraoka *et al.*, 1984) and one γ-tubulin (*gtb1*$^+$, Horio *et al.*, 1991) encoding genes have been isolated and analysed. Although the overall genomic organization of these tubulin genes is very similar in fission and budding yeasts (Neff *et al.*, 1983; Schatz *et al.*, 1986; Sobel and Snyder, 1996), the role of microtubules appears different. Unlike budding yeast, where microtubules are involved mainly in mitosis and meiosis (Reijo *et al.*, 1994), in fission yeast, as in other eukaryotes, microtubules are crucial for more diverse processes. These include cell morphogenesis, growth polarity and the distribution of organelles such as mitochondria (Umesono *et al.*, 1983a; Hiraoka *et al.*, 1984; Yaffe *et al.*, 1996; Mata and Nurse, 1997).

In order to identify more genes and products which are required for microtubular functions, we screened for temperature-sensitive mutants which are defective in cell polarity (Yaffe *et al.*, 1996). One of the loci whose mutation results in the loss of microtubules at the restrictive temperature encodes a protein (termed Alp1, see below) homologous to the mammalian cofactor D. Here we show that Alp1 executes a function essential for cell viability. Analyses of the Alp1 protein have led us to propose that, in addition to a function in the folding of tubulin molecules, cofactors may play a vital role as microtubule-associated proteins *in vivo*.

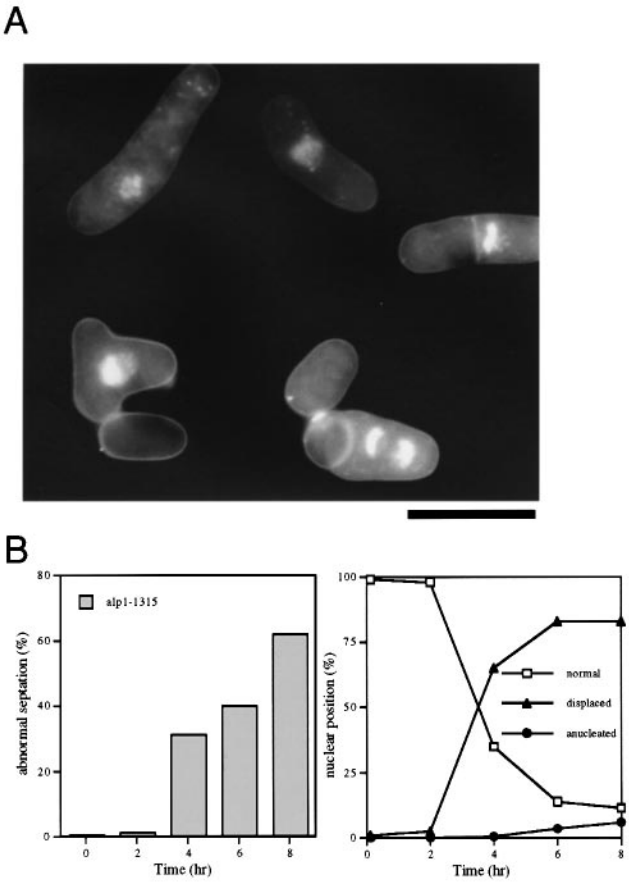

Fig. 1. The *alp1* mutant is defective in growth polarity, mitosis, positioning of the nucleus and symmetrical cell division. (**A**) *alp1-1315* cells grown in rich YPD media at 26°C were shifted to 36°C and incubated for 6 h. Chromosomal DNA was stained with DAPI. The bar indicates 10 μm. (**B**) The percentage of cells with an abnormal septum (left) or nuclear position (right) is indicated at different times after temperature shift.

Results

Isolation of temperature-sensitive polarity mutants

In order to understand the fundamental mechanisms that determine cell polarity, we have visually screened for fission yeast mutants with altered polarity. From a collection of temperature-sensitive (ts) mutants, 15 loci (*alp1*–*alp15*, altered polarity) were identified. *alp1* is defined by three different alleles and represents the most frequently mutated locus. Incubation of *alp1-1315* cells at the restrictive temperature resulted in many cells exhibiting either bent or curved morphologies; altered morphology became apparent after 4 h and increased afterwards (32% after 4 h and 62% after 8 h at 36°C, Figure 1A, upper cell) instead of the normal linear cylindrical shape, and sometimes branched cells were observed (6%, lower left). Notably, in ~80% of the cells, the nucleus was displaced from the centre of the cell (Figure 1A and B). In addition, septum deposition was not central, leading to anucleate cells caused by asymmetrical cell division (Figure 1A, lower two cells and 1B). Nuclear morphology stained with 4′,6′-diamidino-2-phenylindole (DAPI) showed a high frequency (55%, upper left) of cells with condensed chromosomes reminiscent of mitotic figures (Umesono *et al.*, 1983a), indicating that mitosis is retarded in the *alp1-1315* mutant.

Microtubule structures become highly unstable in the alp1 mutant

The phenotypes described above suggest that the *alp1-1315* mutant has defects in cytoskeletal structures. To examine this possibility, indirect immunofluorescence microscopy was performed with anti-tubulin antibodies. Microtubules in the *alp1-1315* mutant appeared fragile or destabilized. At the restrictive temperature, punctate staining, rather than the normal filamentous arrays (which were observed at the permissive temperature), was apparent (Figure 2A and B). To exclude the possibility of artefactual loss of microtubules in the processing of the cells, a mixing experiment was performed where ts *alp1* cells were fixed and processed in the same tube as those of a ts *cdc25-22* mutant blocked at G2 phase (Nurse, *et al.*, 1976). As shown in Figure 2C, long interphase microtubule arrays were observed in the longer *cdc25-22* cell (upper long cell), whilst no microtubule staining was obtained in *alp1-1315* cell (lower bent cell). Disruption of microtubule structures in the *alp1* mutant started immediately after shifting to 36°C, and preceded the displacement of the nucleus (compare Figures 1B and 2D). The kinetics of the disappearance of microtubules in the ts *alp1* mutant are in fact very similar to those in the ts *atb2*/*alp2* mutant that is defective in the α-tubulin gene

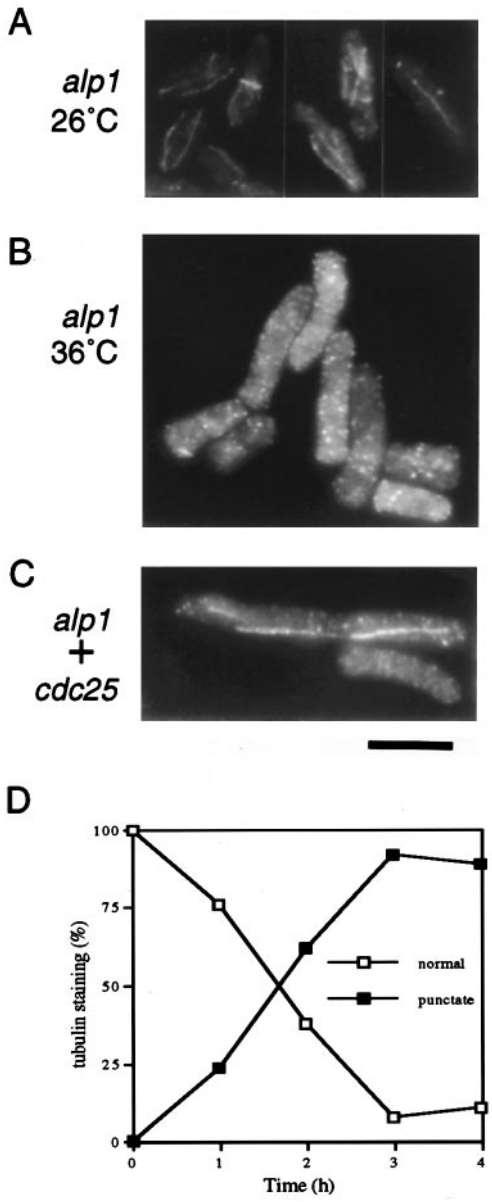

Fig. 2. Microtubule structures are disrupted in the *alp1* mutants. (A and B) *alp1-1315* cells grown as in Figure 1 were fixed and stained with anti-tubulin antibody (TAT-1) before (**A**, 26°C) and after (**B**, 36°C) shift up. (**C**) The *alp1* mutant and the G2 arrest *cdc25* mutant incubated at 36°C for 4 h were mixed and processed for immunofluorescence microscopy. The elongated cell (upper) with long cytoplasmic microtubules is *cdc25*-22, and the bent cell (lower) with a punctate pattern is *alp1-1315*. The bar indicates 10 μm. (**D**) The appearance of microtubule structures was scored after temperature shift.

(Yaffe *et al.*, 1996). This suggests that the disappearance of normal microtubules is a primary reason for the polarity defects in the *alp1* mutant.

Alp1$^+$ *encodes a fission yeast homologue of mammalian cofactor D*

The *alp1*$^+$ gene was cloned by complementation (Materials and methods). The open reading frame (ORF) contains five putative introns (see the legend to Figure 3). The predicted protein consists of 1121 amino acid residues and is homologous to bovine cofactor D (Tian *et al.*, 1996) and to an ORF identified by the *Caenorhabditis elegans* sequencing project (F16D3.4) (28% identity and 44% homology if conservative changes are considered, Figure 3). It is also more weakly related to budding yeast Cin1 (18% identity, Hoyt *et al.*, 1990; Stearns *et al.*, 1990). Cofactor D has been shown to be required for the production of assembly-competent β-tubulins and, in concert with cytoplasmic chaperonin (CCT/TriC/c-cpn), to help in stabilizing β-tubulins (Frydman *et al.*, 1992; Yaffe *et al.*, 1992; Sternlicht *et al.*, 1993; Tian *et al.*, 1995, 1996). Cin1, although non-essential at normal growth temperature, is also involved in microtubule-related pathways (Hoyt *et al.*, 1990; Stearns *et al.*, 1990). Gene disruption experiments showed that the *alp1*$^+$ gene is essential for cell viability (Materials and methods).

The following results provide further support for the notion that Alp1 is involved in microtubule-mediated functions. Firstly, the defective phenotypes of the *alp1* mutant are similar to those found in strains with mutations in the tubulin genes where bent or branched cells are observed (Umesono *et al.*, 1983a,b; Hiraoka *et al.*, 1984; Yaffe *et al.*, 1996). Secondly, as with many mutations in the tubulin genes (Umesono *et al.*, 1983b; Adachi *et al.*, 1986), *alp1* mutants are supersensitive to anti-tubulin drugs even at the permissive temperature; they are incapable of forming colonies on plates containing 20 μg/ml of thiabendazole at 26°C. Taken together, these results show that microtubules are vital to direct polarized growth and to position the nucleus and the septum in the centre of the cell in fission yeast, and that Alp1 is required for these processes.

Overexpression of alp1$^+$ *is toxic, causing altered cell polarity, and is rescued by co-expression of the β-tubulin gene*

To examine the phenotypic consequence of ectopic overexpression of the *alp1*$^+$ gene, the entire *alp1*$^+$ gene was cloned under the thiamine-repressible *nmt1* promoter (Materials and methods; Maundrell, 1990). Transformants were selected on minimal medium containing thiamine and then transferred to medium without thiamine. As shown in Figures 4 and 5A, overexpression of the *alp1*$^+$ gene is lethal; cell division arrested and no colonies were formed in the derepressed condition (Figure 4A, right, lower left). Alp1-overproducing cells often elongated with bent or curved morphology, and microtubule structures appeared abnormal (Figure 4B, and see below), suggesting that an excess amount of cofactor D impairs microtubule-dependent processes. We estimated that a >30-fold overproduction of the Alp1 protein was achieved in these cells (Figure 5B).

It has been shown that cofactor D binds β-tubulin molecules *in vitro* (Tian *et al.*, 1996). To investigate whether co-expression of β-tubulin molecules has any effect on the lethality caused by overexpression of Alp1, two different plasmids, one containing *alp1*$^+$ and the other *nda3*$^+$ (β-tubulin), both under the *nmt1* promoter, were expressed simultaneously. Interestingly, plasmid containing *nda3*$^+$ was capable of rescuing toxicity by overexpression of *alp1*$^+$ (Figure 4A, lower right). This result suggests that, consistent with biochemical data, cofactor D binds β-tubulin monomer in the cell.

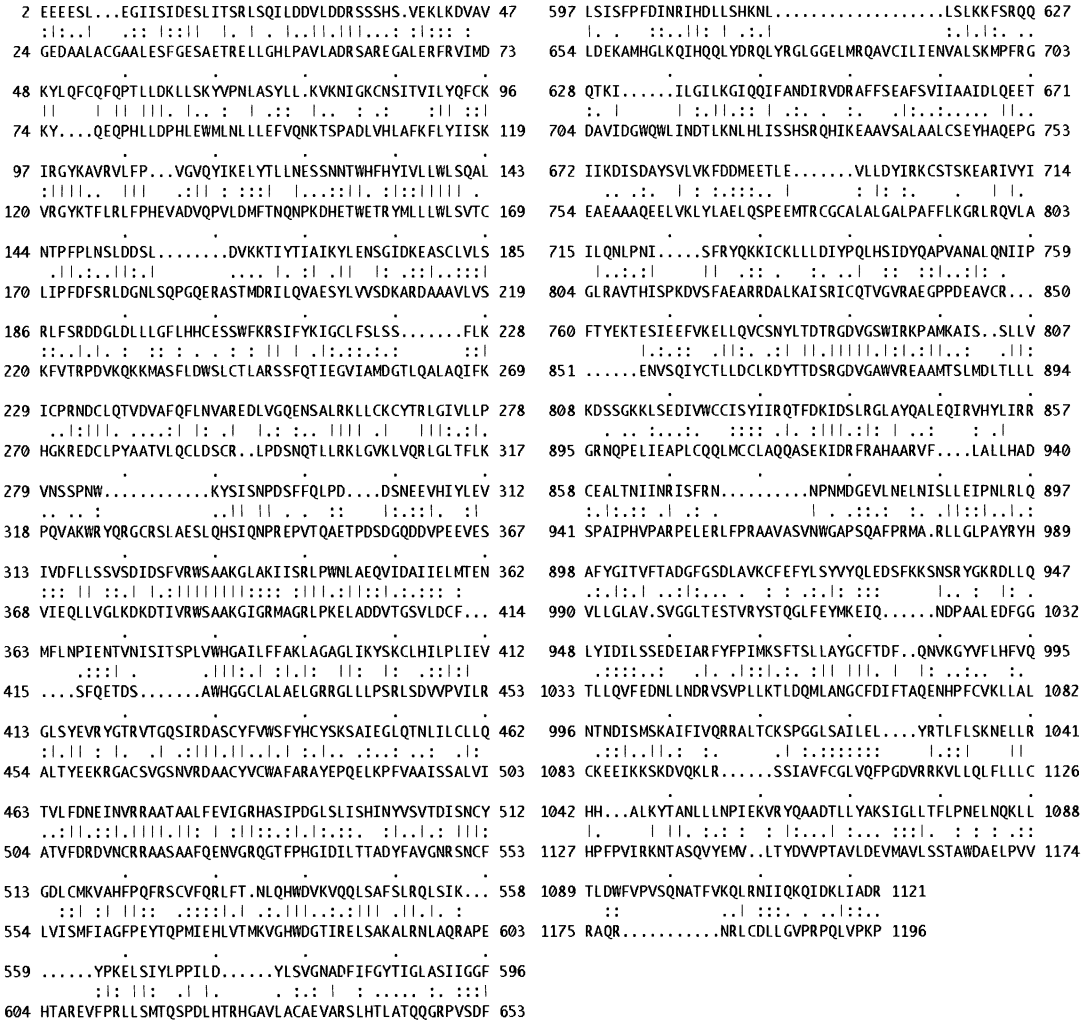

Fig. 3. Comparison of amino acid sequences of Alp1 and cofactor D. The upper line is Alp1, the lower is bovine cofactor D. Amino acid comparison was done using the BestFit program. The sequence of cofactor D is taken from Tian *et al.* (1996). Amino acids 988–1011 (YVFLHFVQNINDIS) might not exist in the Alp1 protein, as a consensus sequence for *S.pombe* introns (in-frame) is found in this region.

Alp1 co-localizes with microtubules

To determine the cellular localization of the Alp1 protein, the *alp1*$^{+}$ gene was cloned in-frame with a fragment encoding green fluorescent protein (GFP) from jellyfish (Chalfie *et al.*, 1993). The addition of GFP to Alp1 does not interfere with the function of Alp1, as GFP–Alp1 is capable of rescuing ts *alp1* and also the deletion of the *alp1*$^{+}$ gene (Materials and methods). GFP–Alp1, like Alp1, was toxic when massively overexpressed, but the addition of a low concentration of thiamine to the media efficiently repressed the expression of GFP–Alp1 (Toda *et al.*, 1996). As shown in Figure 5B, from an ~30-fold overexpression in the absence of thiamine (lanes 1 and 4), a 3-fold overexpression was observed in the presence of 50 nM thiamine (lanes 2). This amount of GFP–Alp1 was not toxic; cells continued to grow normally (Figure 5A).

Green fluorescence in cells where GFP–Alp1 was expressed modestly showed that it localized in a filamentous structure in interphase cells (Figure 6, upper). Furthermore, nuclear spindle-like staining was observed in mitotic cells (lower; note the condensed chromosomes). To show clearly the co-localization between Alp1 and microtubules, microtubules were depolymerized using a cold shock treatment and then reversed by transferring the cells into warm medium (Mata and Nurse, 1997). Upon cold shock, no microtubules were observed and GFP–Alp1 did not show any filamentous structures (Figure 7, panel 1). After reversal to 25°C, it is clear that the GFP–Alp1 protein co-localized with both cytoplasmic (panel 2) and mitotic spindles (panels 3 and 4).

The green fluorescence of GFP–Alp1 was observed in cells where GFP–Alp1 was massively overexpressed. Green fluorescence showed that microtubule array-like structures appeared globular and tangled (Figure 4B), suggesting a disorganization of normal microtubule structure and distribution. In these cells, no microtubule structures were observed with immunofluorescence using anti-tubulin antibody (data not shown).

Alp1 co-sediments with porcine brain microtubules

The aforementioned results strongly suggest that Alp1 binds microtubules. To address this further, the microtubule-binding ability of Alp1 was examined as follows (Figure 8A). Fission yeast cell extracts where the Alp1

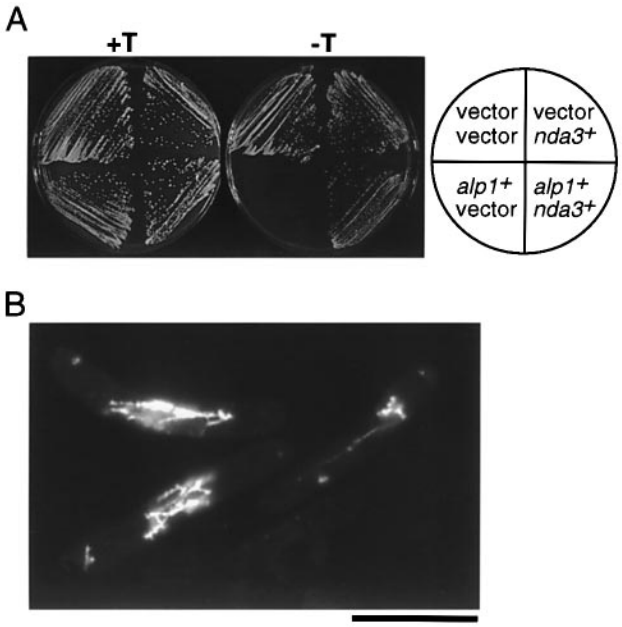

Fig. 4. Lethal overexpression of Alp1 and suppression of lethality by co-expression of β-tubulin. (**A**) Wild-type cells were co-transformed with the following combinations of plasmids and streaked on minimal plates in the presence (left, +T, repressed) or absence (right, –T, derepressed) of thiamine; vectors (pREP1 and pREP2, upper left), pREP1-GFP-*alp1*$^+$ and a vector (lower left), a vector and pREP2-*nda3*$^+$ (lower right) and pREP1-GFP-*alp1*$^+$ and pREP2-*nda3*$^+$. (**B**) GFP–Alp1 was induced by removing thiamine from the medium and, after 16 h, the cells were fixed to observe the green fluorescence. The bar indicates 10 μm.

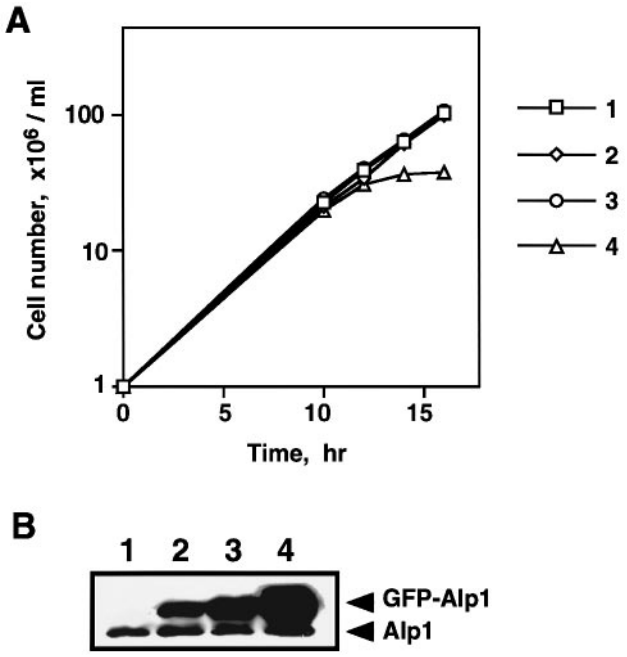

Fig. 5. Expression of Alp1 under the *nmt1* promoter. (A and B) Wild-type cells containing GFP–*alp1*$^+$ were grown in minimal medium in the presence of thiamine (1 μM) at 25°C, filtered, resuspended in minimal medium containing various concentrations of thiamine and incubated at 25°C. Cell number was measured at each time point (**A**) and, after 14 h incubation, protein extracts were prepared and immunoblotting was performed with anti-Alp1 antibody (**B**). Polyclonal rabbit anti-Alp1 antibody was prepared against synthetic peptides (RNIIQKQIDKLIADR) corresponding to the C-terminal region of Alp1 and affinity purified. Concentrations of thiamine used are as follows, 1 μM (□ and lane 1), 50 nM (◇ and lane 2), 25 nM (○ and lane 3) and 0 nM (△ and lane 4). Diamonds and circles are not visible due to overlap with squares.

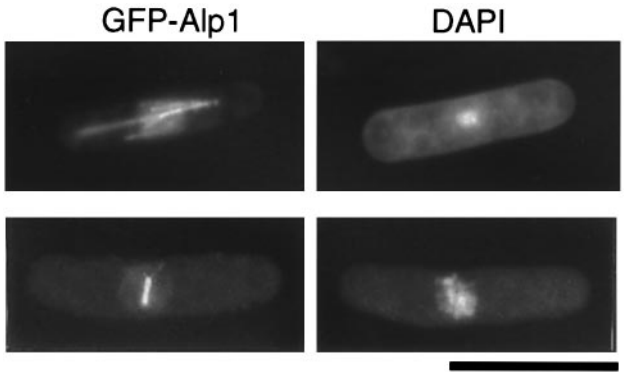

Fig. 6. Alp1 localizes to microtubule-like structures. Wild-type cells carrying GFP–*alp1*$^+$ were grown in media containing a low concentration of thiamine (50 nM) in which pREP1-GFP-*alp1*$^+$ can rescue *alp1*-deleted cells without any harmful effects (Toda *et al.*, 1996). Cells were fixed, stained with DAPI and observed with fluorescence microscopy to visualize GFP (left) and chromosomes (right).

protein was overexpressed (5-fold overexpression compared with a single chromosomal copy, a level similar to that in lane 3 of Figure 5B) were incubated with taxol (paclitaxel)-stabilized microtubules prepared from porcine brain. Mixtures were overlaid on a 60% glycerol cushion and ultracentrifuged to separate microtubule-containing pellet from soluble supernatant. The Alp1 protein co-sedimented with microtubules and its amount in the pellet increased proportionally as more microtubules were added in the mixture (Figure 8B, lanes 2–4 and 7–9). If no microtubules were added, Alp1 existed exclusively in the supernatant fraction (lanes 1, 5, 6 and 10). These data suggest that Alp1 binds microtubules *in vivo*, although it is currently not clear whether Alp1 itself binds directly to microtubules or via other cofactors which interact with cofactor D/Alp1.

Discussion

In this study, we have shown that a fission yeast homologue (Alp1) of cofactor D is required for microtubule-dependent functions such as nuclear division, growth polarity and the positioning of both nucleus and septum. In the ts *alp1* mutant, microtubules become destabilized and disappear, which results in the various defective phenotypes described above. This may be ascribed to a loss of assembly-competent β-tubulin pools in the *alp1* mutant at the restrictive temperature, as biochemical data show that cofactor D is required for the correct folding of β-tubulin (Tian *et al.*, 1996, 1997). Given the co-localization and co-sedimentation of the Alp1 protein with microtubules, however, we would like to point out that cofactors might have another role, in addition to tubulin-folding accessory factors, as microtubule-associated proteins (MAPs) in the cell (see below).

Essential roles of cofactor D in cell viability

Extensive biochemical work has shed light on the importance of cofactors in the process of tubulin folding, at least

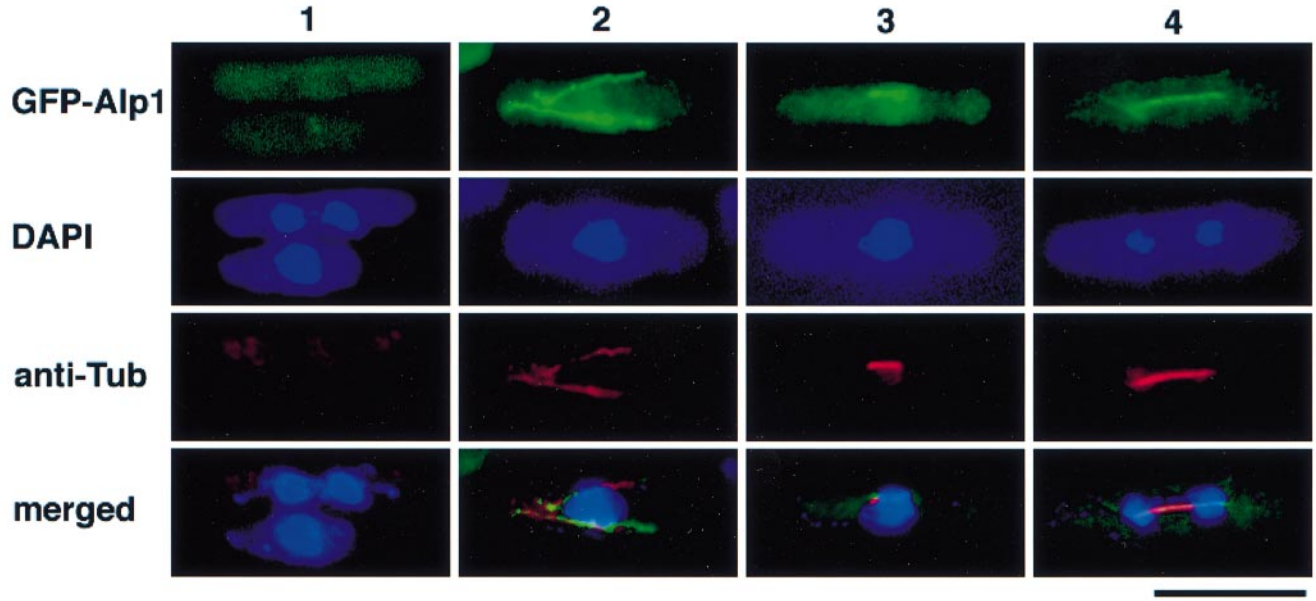

Fig. 7. Alp1 co-localizes with microtubule structures. The same cells as in Figure 6 were incubated on ice for 30 min to disrupt microtubuless and then returned to 25°C to regenerate the microtubuless. After 45 min at 25°C, cells were fixed and processed for immunofluorescence microscopy. GFP (top row), DAPI (second row), anti-tubulin (third row) and merged (bottom row) are shown. Cells incubated on ice are shown as a control (panel 1). After shift-up, interphase cells with cytoplasmic microtubules (panel 2) and mitotic cells with short (panel 3) or long (panel 4) nuclear spindles are observed. The bar indicates 10 μm.

in an *in vitro* system of animal cells (Gao *et al.*, 1993; Rommelaere *et al.*, 1993). In contrast, however, genetic analysis performed in budding yeast suggests that, surprisingly, the cofactor homologues, mutations of which lead to microtubular defects only at low temperature, are dispensable at normal growth temperature, these cofactors are not required under normal growth conditions (Hoyt *et al.*, 1990, 1997; Stearns *et al.*, 1990; Archer *et al.*, 1995; Tian *et al.*, 1997). However, results presented in this study show that this is not the case; rather cofactor D is absolutely required for cell viability and the maintenance of the microtubule architecture in the cell. We recently have undertaken the genetic analysis of other *alp* mutants and isolated homologues of cofactors B and E. Consistent with *alp1*$^+$, disruption of these two homologues is lethal (P.Radcliffe and T.Toda, unpublished results). Therefore, in fission yeast, and probably in mammalian cells also, cofactors have indispensable roles in microtubule biogenesis. We attempted to rescue the *alp1* mutant with mammalian cofactor D (provided by N.J.Cowan and S.Lewis), but were not able to make the necessary constructs and so are unable to address this question. It appears that cofactor D in fission yeast expression plasmids is toxic in bacteria (D.Hirata and T.Toda, unpublished results).

Lethal overexpression of Alp1/cofactor D

A recent biochemical study shows that an excess amount of cofactor D leads to disruption of the α/β tubulin heterodimer by sequestering the β-tubulin subunit (Tian *et al.*, 1997). This phenomenon might be parallel to our observation that overexpression of Alp1 is lethal in fission yeast which leads to no microtubule structures being observed (Figure 4B; D.Hirata and T.Toda, unpublished results). It is, however, also possible that the excess Alp1 binds microtubules tightly, thereby preventing access of the anti-tubulin antibody to the microtubule structures. Ultrastructural observation of microtubules in Alp1-overproducing cells would clarify this point.

The suppression of the lethality of overproduction of Alp1 by co-expression of β-tubulin is intriguing. A similar, but not identical, suppression phenomenon was reported previously in budding yeast where toxic overexpression of the β-tubulin gene (*TUB2*) is rescued by co-expression of the cofactor A-encoding gene (*RBL2*, Archer *et al.*, 1995). Previously, overexpression of β-tubulin was reported to be toxic in both budding and fission yeast cells (Hiraoka *et al.*, 1984; Schatz *et al.*, 1986; Matsuzaki *et al.*, 1988; Burke *et al.*, 1989; Katz *et al.*, 1990). We have not, however, observed toxicity by Nda3 overexpression in this study (Figure 4A), probably due to differences in copy number of the plasmids used. We used a *ura4*$^+$- and *ars1*-based plasmid instead of the *LEU2* and 2 μm plasmid which was used before (Hiraoka *et al.*, 1984).

Does Alp1/cofactor D act as an MAP?

Of particular interest is the observation that Alp1 co-localizes with microtubules. If Alp1/cofactor D were only required for the folding of β-tubulin, Alp1 might be expected to be localized in the cytoplasm. Although GFP–Alp1 is overexpressed compared with chromosomal Alp1, it is functional, and the level of expression is sufficiently modest (3-fold) for it not to interfere with normal growth. Attempts to localize the chromosomally derived Alp1 protein using anti-Alp1 antibody have so far been unsuccessful. Nevertheless, we show that the Alp1 protein, when incubated with porcine brain microtubules, co-sediments with them. These results strongly suggest that a proportion of Alp1 protein binds and exists along microtubules in the cell. Whether Alp1 binds microtubules directly or indirectly remains to be determined. In view of this, it should be noted that cofactors B and E, but not

D, contain a potential microtubule-binding motif that is found in several MAPs, including CLIP-170/restin, Glued and Bik1 (Rickard and Kreis, 1996; Tian *et al.*, 1996, 1997), and that cofactors C and E behave biochemically as MAPs at least *in vitro* (Tian *et al.*, 1996). Thus, it is possible that complexes of cofactors act as MAPs.

It could be speculated that cofactors have a dual function: not only are they involved in the chaperonin-mediated protein folding of tubulin monomers but they also act as MAPs to regulate the dynamics of microtubules directly. This implies that tubulin folding and assembly steps are also important for stabilizing microtubule structures. Furthermore, it is also possible that the intracellular level of free cofactors is regulated through an interaction between cofactors and microtubules (Tian *et al.*, 1996). In this scenario, microtubules and cofactors comprise a functional feedback loop. On the one hand, cofactors regulate microtubules through tubulin folding and probably as microtubule-stabilizing MAPs, and on the other, the amount of microtubules regulates the level of free cofactors which interact with chaperonin for the assembly of tubulins. In any case, cofactors are indispensable for the *in vivo* dynamics of microtubule-dependent processes.

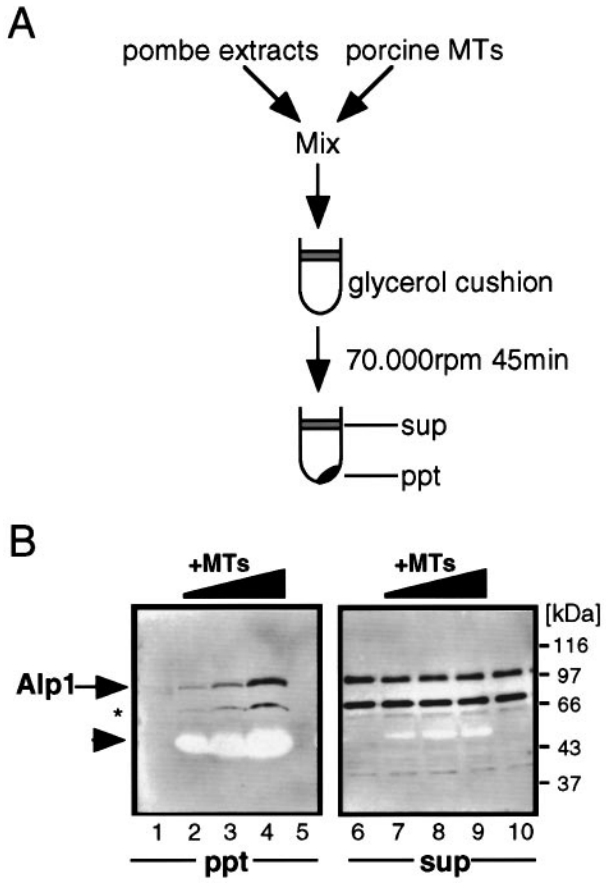

Fig. 8. Microtubule-binding assay of the Alp1 protein.
(A and B) Schematic presentation of the experiment (**A**) and immunoblotting with anti-Alp1 antibody (**B**) are shown. *Schizosaccharomyces pombe* cell extracts were prepared and mixed with different concentrations of taxol (paclitaxel)-stabilized porcine brain microtubules (lanes 1, 5, 6 and 10, 0 μM; lanes 2 and 7, 20 μM; lanes 3 and 8, 40 μM; lanes 4 and 9, 60 μM). Pellet (ppt) and supernatant (sup) were separated by centrifugation through a glycerol cushion (800 μl), and an equivalent amount of each fraction was separated by SDS–PAGE and immunoblotted with anti-Alp1 antibody (ppt, lanes 1–5; sup, lanes 6–10). The Alp1 protein was recognized as a 100 kDa band (arrow). The band of ~70 kDa (asterisk) might be a degraded form of Alp1 as it behaves in the same manner as $p100^{alp1}$. The position of tubulins is shown by an arrowhead, and are also recognized as white bands (lanes 2–4 and 7–9). Size markers are shown on the right.

Materials and methods

Isolation of temperature-sensitive mutants with polarity defects

Wild-type cells (HM123, *h⁻leu1-32*, Table I) were mutagenized with *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine as described previously (Uemura and Yanagida, 1984). In total, 200 960 colonies were screened for temperature sensitivity, and 2822 ts mutants were isolated. Mutants which showed altered cell shape were then selected visually by fluorescence microscopy after staining with Calcofluor white (Sigma). Twenty-two ts mutants which had bent, curved or branched morphologies were isolated and designated *alp* (altered polarity). Complementation tests showed that these mutants defined 15 loci. *alp1* consists of three alleles (*alp1-1315*, *1356*, *1584*). DH2-8D (*h⁻leu1alp1-1315*, Table I) was used in this study.

Cloning of the $alp1^+$ gene

The ts *alp1* strain was transformed with a *Schizosaccharomyces pombe* genomic library using the lithium method (Beach *et al.*, 1982; Ito *et al.*, 1983). From 36 000 transformants examined, two were capable of forming colonies at 36°C. The plasmids recovered were identical and designated pAL100, and the insert DNA was shown to be derived from the $alp1^+$ locus by integration. The nucleotide sequence data is available in the DDBJ/EMBL/GenBank databases under accession No. Y10106.

Gene disruption

A 4.7 kb *Bgl*II–*Sph*I fragment containing the $alp1^+$ gene was cloned into pUC18, and an internal 1.2 kb *Pst*I–*Hpa*I fragment, corresponding to amino acid residues 330–741, was replaced with the 1.8 kb $ura4^+$ cassette (Grimm *et al.*, 1988). The 5.3 kb $alp1::ura4^+$ fragment was used to transform the diploid strain CHP428/429 (Table I). Stable Ura^+ colonies were picked, and correct disruption was checked by Southern hybridization. Tetrad analysis indicated that the $alp1^+$ gene is essential for viability; two viable Ura^- (and two non-viable Ura^+) colonies were obtained from 20 tetrads dissected.

Plasmid construction

The entire ORF of the $alp1^+$ or $nda3^+$ gene was amplified by PCR and cloned such that it was under the control of the thiamine-repressible *nmt1* promoter [pREP1-$alp1^+$ or pREP2-$nda3^+$ (Maundrell, 1990)]. To construct the GFP-tagged $alp1^+$ gene, a PCR-amplified fragment encoding GFP was inserted in-frame prior to the initiation codon of $alp1^+$ in

Table I. Strain list

Strain	Genotype	Source
HM123	*h⁻leu1*	our stock
cdc25	h^+*cdc25-22*	from Paul Nurse
DH2-8D	*h⁻leu1alp1-1315*	this study
TP108-3A	*h⁻leu1ura4*	our stock
CHP428/429	h^-/h^+*leu1/leu1ura4/ura4his7/his7 ade6-210/ade6-216*	from C.Hoffman
ME1	h^-/h^+*leu1/leu1ura4/ura4his7/his7 ade6-210/ade6-216alp1::ura4*$^+$/+	this study

pREP1-*alp1*$^+$ (pREP1-GFP-*alp1*$^+$). Both pREP1-*alp1*$^+$ and pREP1-GFP-*alp1*$^+$ were functional as they complemented the ts *alp1-1315* mutation and also rescued the *alp1* disruptant in the presence of thiamine. pREP2-*nda3*$^+$ is also functional as it suppresses the ts *nda3*/*alp12* mutant (P.Radcliffe and T.Toda, unpublished results). TP108-3A (*h*$^-$ *leu1ura4*, Table I) was used for double transformation.

Immunological methods

For immunofluorescence microscopy, TAT-1 antibody (provided by Dr Keith Gull) and Cy3-conjugated sheep anti-mouse IgG (Sigma) were used to visualize microtubules. Polyclonal rabbit anti-Alp1 antibody was prepared against synthetic peptides (RNIIQKQIDKLIADR) corresponding to the C-terminal region of Alp1 and affinity purified. Immunoblots of affinity-purified anti-Alp1 against total cell extracts detected a band of ~100 kDa whose intensity increased in *alp1*$^+$-overproducing cells, showing that p100 is the *alp1*$^+$ gene product.

Cold shock experiment

Wild-type cells containing pREP1-GFP-*alp1*$^+$ were grown in minimal medium containing 50 nM of thiamine at 25°C for 14.5 h. Cells were incubated on ice for 30 min, returned to the same medium at 25°C for 45 min, fixed and processed for immunofluorescence microscopy.

Microtubule-binding assay

Porcine brain microtubules were prepared essentially as described (Masuda and Cande, 1987). Fission yeast cells containing pREP1-*alp1*$^+$ grown at 26°C for 14 h in the derepressed condition (in the absence of thiamine) were washed with ice-cold STOP buffer (150 mM NaCl/ 50 mM NaF/10 mM EDTA/1 mM NaN_3) and disrupted with glass beads in DB buffer (25 mM MOPS pH7.2/60 mM β-glycerophosphate/15 mM $MgCl_2$/15 mM EGTA/0.1% NP-40/0.1 mM sodium vanadate/a cocktail of protease inhibitors). Extracts (2 mg/ml) were centrifuged at 15 000 r.p.m. for 10 min at 4°C and stored at –80°C. Soluble extracts were prepared further as follows. GTP and dithiothreitol (DTT) (1 mM, respectively) were added to frozen extracts and centrifuged at high speed (80 000 r.p.m. for 30 min at 4°C) and supernatant was used immediately for the assays. Sixty-five μl of soluble extracts were mixed with 60 μl of BRB80-1 [80 mM PIPES pH6.8/1 mM $MgCl_2$/1 mM EGTA/1 mM DTT/20 μM taxol (paclitaxel)/a cocktail of protease inhibitors] containing various amounts of microtubules (0–60 μM) and 1 mM GTP and incubated at room temperature for 10 min. Mixtures were overlaid onto a cold 60% glycerol cushion in BRB80-2 [the same as BRB80-1 except that it contains 10 μM taxol (paclitaxel)] and centrifuged at 70 000 r.p.m. for 45 min at 4°C. Pellet and supernatant were separated, and each sample was run on SDS–PAGE, immunoblotted with anti-Alp1 antibody and visualized by the ECL system (Amersham).

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