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Tubulin heterodimers remain functional for one cell cycle after the inactivation of tubulin-folding cofactor D in fission yeast cells

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Abstract

Tubulin-folding cofactor D plays a major role in the formation of functional tubulin heterodimers, the subunits of microtubules (MTs) that are essential for cell division. Previous work has suggested that, in *Schizosaccharomyces pombe*, cofactor D function is required during G₁ or S phases of the cell cycle, and when it fails to function due to the temperature-sensitive mutation *alp1-t1*, cells are unable to segregate their chromosomes in the subsequent mitosis. Here we report that another mutation in the cofactor D gene, *alp1-1315*, causes failures in either the first or second mitosis in cells synchronized in G₁ or G₂ phases, respectively. Other results, however, suggest that the kinetics of viability loss in these mutants does not depend on progression through the cell cycle. When cofactor D function is perturbed in cells blocked in G₂, cytoplasmic MTs appear normal for 2–3 h but thereafter they disintegrate quickly, so that only a few short MTs remain. These residual MTs are, however, stably maintained, suggesting that they do not require active cofactor D function. The abrupt disassembly of MT cytoskeleton at restrictive temperature in non-cycling cofactor D mutant cells strongly suggests that the life-span of folded tubulin dimers might be downregulated. Indeed, this period is significantly shorter than the previously determined dissociation time of bovine tubulins *in vitro*. The death of mutant cells occurs inevitably after 2–3 h at restrictive temperature in the following mitosis, and is explained by the idea that MT structures formed in the absence of cofactor D cannot support normal cell division.

Keywords

microtubule; *Schizosaccharomyces pombe*; chromosome segregation; tubulin dimers

Introduction

Microtubules (MTs) participate in many cellular processes including protein and mRNA transport, cell motility and morphogenesis (reviewed in Beghin *et al.*, 2007). They become essential in mitosis, when they contribute to the formation of the spindle, a MT-based

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apparatus required for proper chromosome segregation (reviewed in McIntosh *et al.*, 2002; Walczak and Heald, 2008). MTs are polymers of $\alpha\beta$ -tubulin heterodimers, two related proteins whose genes are expressed throughout the cell cycle (Yokota *et al.*, 1999). The elevated concentration of β -tubulin is highly toxic (Hiraoka *et al.*, 1984; Weinstein and Solomon, 1990), and the intracellular levels of both α - and β -tubulins remain constant throughout the cell cycle (Katz *et al.*, 1990). This is achieved in part by an autoregulatory mechanism, which leads to a specific degradation of tubulin mRNAs if the intracellular concentration of tubulin subunits increases (Cleveland *et al.*, 1981).

Another mechanism of controlling the availability of tubulin dimers is carried out via an elaborate tubulin-folding pathway. Newly synthesized tubulin polypeptides are first 'pre-folded' by cytosolic chaperonin; in mouse and human cells, this occurs predominantly in early S-phase (Yokota *et al.*, 1999). The pre-folded α - and β -tubulins, however, require additional processing to form functional $\alpha\beta$ -heterodimers. This is achieved presumably continuously during the cell cycle by a specialized biochemical pathway consisting of five proteins, named tubulin-folding cofactors A–E (reviewed in Lopez-Fanarraga *et al.*, 2001; Szymanski, 2002). *In vitro*, α - and β -tubulins are first sequestered by cofactors B and A, respectively, and then transferred to cofactors E and D (Tian *et al.*, 1996; Tian, 1997). Tubulin folding cofactor D appears to play the role of a 'scaffold', which assembles both tubulins and cofactors E and C into a super-complex (Grynberg *et al.*, 2003). Binding of the latter protein facilitates the GTP-dependent release of the fully functional $\alpha\beta$ -tubulin heterodimer (Tian *et al.*, 1999).

Removal of human cofactor D from HeLa cells using siRNA did not affect density of cytoplasmic MTs (Cunningham and Kahn, 2008). However, in the fission yeast *S. pombe* and the plant *Arabidopsis thaliana*, the tubulin-folding pathway is essential for cell viability and, in the absence of cofactors, the MT cytoskeleton is greatly perturbed and diminished (Grishchuk *et al.*, 1998; Grishchuk and McIntosh, 1999; Hirata *et al.*, 1998; Radcliffe *et al.*, 1999, 2000; Steinborn *et al.*, 2002). Thus, it is plausible that when the intracellular folding pathway is disrupted, the newly synthesized tubulins remain unfolded and cannot function normally. It is unclear, however, how long already folded tubulin dimers can function in the absence of this pathway *in vivo*. *In vitro*, the half-time for dissociation of bovine tubulin dimers with GTP-bound nucleotide and in the absence of the cofactors is ~9.6 h (Caplow and Fee, 2002). It has been suggested that the cofactors can catalyse the dissociation of tubulin heterodimers, thereby limiting the lifetime of already formed tubulin dimers (Caplow and Fee, 2002). Consistently, overexpression of bovine cofactor D in HeLa cells leads to reduced level of α -tubulin and MT structures (Bhamidipati *et al.*, 2000; Cunningham and Kahn, 2008; Martin *et al.*, 2000). However, overexpression of human cofactor D does not change tubulin levels in HeLa cells (Cunningham and Kahn, 2008), so cellular roles of cofactor D deserve further study.

The interpretation of the phenotypes of the cofactors' disruptions is therefore complicated by indications that this pathway, or individual cofactor proteins, may play other roles in addition to tubulin folding. As elucidated above, this pathway may be involved in the regulation of the availability of tubulin dimers, thereby affecting the MT dynamics (reviewed in Beghin *et al.*, 2007; Martin *et al.*, 2000; Kortazar *et al.*, 2006, 2007; Tian *et al.*, 1997,

1999). Recent studies have shown that human cofactor D has an additional function: it is required for the recruitment of γ -tubulin ring complex to the centrosome, which is essential for MT nucleation by this organelle (Cunningham and Kahn, 2008).

To gain more insight into the cellular roles of the tubulin-folding pathway, we have used the cofactor D mutants in the fission yeast *Sz. pombe*. All basic features of this pathway in fission yeast appear to be similar to those reconstructed using mammalian proteins *in vitro*, except that the pathway is more linear, with cofactor D acting downstream of cofactors A, B and E, and thus playing a more prominent role (Radcliffe *et al.*, 1999, 2000). It has been shown that both deletion and overexpression of cofactor D lead to the disruption of MT cytoskeleton (Grishchuk *et al.*, 1998; Hirata *et al.*, 1998). These phenotypes suggest that fission yeast cofactor D may play role in both tubulin dimer formation and dissociation of already formed tubulin dimers, resulting in the dispersion of MTs. Furthermore, cofactor D may additionally contribute to organization of the MT cytoskeleton as a microtubule-associated protein (Hirata *et al.*, 1998). It is not known whether cofactor D helps to nucleate MTs in *Sz. pombe*, and whether this or any of its other functions are regulated during the cell cycle.

Two mutant alleles of the tubulin-folding cofactor D gene in fission yeast have been previously studied: *alp1-1315* and *tsm1-512*, also known as *alp1-t1*. Although at the restrictive temperature both mutant strains have severe disruption of the MT cytoskeleton, many details of this and other collateral phenotypes are different in these strains (Fedyanina *et al.*, 2006; Hirata *et al.*, 1998). Based on these differences, we have previously concluded that the *alp1-1315* mutation produces more severe defects at the restrictive temperature than the milder *alp1-t1* allele, although the latter mutation leads to partial impairment of cofactor D function at the permissive temperatures (Fedyanina and Grishchuk, 2004; Fedyanina *et al.*, 2006). We have also shown that, when *alp1-t1* cells are synchronized in G₂ phase of the cell cycle and cells are then transferred to the restrictive temperature, they divide normally once and lose their viability only during the second mitosis (Grishchuk *et al.*, 1998). By analogy with other fission yeast mutants with this cell cycle phenotype, this result suggested that cofactor D may have some function in G₁ or S phases of the cell cycle (Furuya *et al.*, 1998; Saitoh *et al.*, 1997). Alternatively, the delayed development of mutant phenotypes in these cells may be due to a slow kinetics of cofactor D inactivation. Here, we distinguish between these possibilities by examining the protein levels of Alp1-t1 and tubulins, and by using various cell cycle techniques to study the behaviour of the more severe *alp1-1315* mutant allele of cofactor D. Our data suggest that, in the absence of normal cofactor D, fission yeast cells have a significant pool of functional tubulin heterodimers, which, however, becomes depleted after one mitotic division, leading to a catastrophe during the following division.

Materials and methods

Sz. pombe methods

Strains used in this study are listed in Table 1. All media and growth conditions were as described in Moreno *et al.* (1991). Cells were grown either in rich (YES) or minimal (EMM) medium with supplements. Synchronization of strains in early G₂ phase of the cell cycle was

carried out by gradient centrifugation, as described in Grishchuk *et al.* (1998). Synchronization of strains in G₁ phase was carried out by nitrogen starvation, as described in Goshima *et al.* (1999). Briefly, *alp1-1315* mutant cells (without auxotrophic markers) grown in EMM at 25 °C at 5×10^6 cells/ml were washed in EMM–N (EMM medium without the source of nitrogen, NH₄Cl) and cells (2×10^7 cells/ml) were incubated at 25 °C for 22 h. Cells were then released into complete medium (YES) and cultured at 36 °C. The procedure of FACSscan analysis was performed as described (Grishchuk *et al.*, 1998). Briefly, approximately 1×10^7 cells/ml yeast cells were collected, fixed in 70% ethanol and stored at 4 °C for several days. 15–100 µl cell suspension in 70% EtOH was resuspended in 1 ml 50 mM sodium citrate with 1 mg/ml RNase. After 2–4 h of incubation at 37 °C, propidium iodide (Sigma) was added at 5 µg/ml. Stained cells were diluted in 50 mM sodium citrate and briefly sonicated. The DNA content was analysed with a FACSscan (Becton-Dickinson, San Jose, CA, USA). The control was a log-phase haploid wild-type culture. The construction of *cdc10-129 alp1-1315*, *cdc25-22 alp1-1315* and *cdc25-22 alp1-t1* double mutants was performed using random spore analysis. Cell viability was obtained at each time point by plating the cultured cells at 25 °C on YES medium. For cytology analysis, the cells were fixed in 70% ethanol or by heat fixation and stained with 4',6-diamidino-2-phenylindole (DAPI) (Sigma) and Calcofluor (Sigma), as described in Grishchuk *et al.* (1998).

Immunofluorescence

For immunofluorescence, cells were fixed in methanol at –20 °C for 4–8 min. Immunofluorescent staining of MTs was carried out as described (Grishchuk *et al.*, 1998), using primary TAT1 antibodies (kindly provided by Keith Gull, University of Manchester Institute of Science and Technology, UK) and secondary goat anti-mouse antibodies labelled with Alexa 488 (Molecular Probes Inc.). More than 300 cells were counted for each time point. Cells were viewed with a $\times 100$ Plan oil-immersion objective on a Leica DM LA microscope. Images were captured using a Roper Scientific Coolsnap FX camera and processed using the ImageJ v. 1.34s program (fusion Complex Wavelets method).

Western blot analysis

Fission yeast whole extracts were prepared by disrupting cells with glass beads, as described in Alfa *et al.* (1993). Mouse monoclonal anti- α -tubulin antibodies (4A1, used at 1 : 200) were kindly provided by Dr M. Fuller (Stanford University); rabbit monoclonal anti- β -tubulin antibodies (used at 1 : 1000) were kindly provided by Dr H. Masuda (Kansai Advanced Research Centre); mouse monoclonal anti-Cdc2-antibodies (PSTAIRe, used at 1 : 200) were purchased from Santa Cruz Biotechnology. Horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G and horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Bio-Rad Laboratories, Richmond, CA, USA) and a chemiluminescence system (ECL, Amersham, Arlington Heights, IL, USA) were used to detect bound antibody. For quantitative analysis, the integral intensities of bands seen with anti-tubulin antibodies were obtained using Adobe Photoshop CS and normalized based on the values for Cdc2p. The band intensity of wild-type at the zero time point was taken as 100%.

For analysis of Alp1-t1 level, the strain with Alp1-t1- GFP-(Pk1)₃ was constructed (SP 69 in Table 1) as follows. A plasmid vector containing the green fluorescent protein (S65T allele; Heim *et al.*, 1995), followed in-frame by three tandem copies of the Pk1 epitope tag (Southern *et al.*, 1991) and the *ura4⁺* gene was generously provided by B. Carson and C. Troxell (University of Colorado). PCR was used to amplify a continuous ~3.5 kb fragment containing the Pk1 and GFP tags together with the *ura4⁺* gene. For PCR, we followed published protocols (Grishchuk *et al.*, 2000; Innis *et al.*, 1990). The GFP-Pk1-*ura4⁺* PCR product was used directly to transform cells (SP 26), and Ura⁺ colonies were selected for further analysis. Mouse monoclonal anti-PK1 antibodies (Novus Biologicals Inc.) were used at 1 : 500 dilution for detection of Alp1-t1; mouse monoclonal antibody against glucose 6-phosphate dehydrogenase was used to obtain a loading control. For quantitative analysis, the integral intensities of bands were obtained using Adobe Photoshop CS and normalized on the values for glucose 6-phosphate dehydrogenase. The band intensity at zero time point was taken as 100%.

Results

Levels of α - and β -tubulins remain unchanged in the cells with mutant cofactor D

When asynchronous culture of *alp1-t1* cells is shifted to the restrictive temperature (36 °C), cells lose their viability rather slowly but, after 6 h, only 15% of cells remain viable (Grishchuk *et al.*, 1998). To examine whether this result reflected a slow kinetics of inactivation of mutant cofactor D, we collected samples from *alp1-t1* cultures at different times after the temperature shift and examined the levels of relevant proteins by separating them on SDS gel, followed by Western blotting and antibody application. To analyse the levels of cofactor D, we created a C-terminal fusion of the *alp1-t1* gene at its endogenous location with green fluorescent protein (GFP) and three tandem copies of the Pk1 epitope tag (see Materials and methods). Since the expression of this fusion gene is driven by a normal promoter of the *alp1-t1* gene, the level of its product is likely to be similar to those of the *alp1-t1* gene product without the fusion. Figure 1A shows that the level of Alp1-t1 protein decreases at 36 °C, and by 6 h it falls to ~30% of its level at the permissive temperature (25 °C).

To examine whether these changes led to a concomitant reduction in tubulin levels, we used the untagged *alp1-t1* cells and probed the blots with antibodies to β - or α -tubulins (Figure 1B). The latter antibody recognizes the products of two related α -tubulin genes, but their corresponding bands are positioned closely, so we combined these signals together. At 25 °C the levels of β - and combined α -tubulins were noticeably lower in *alp1-t1* cells than in wild-type cells, consistent with our previous finding that this mutation impairs cofactor D function at this temperature. However, even 5.5 h of incubation of this strain at 36 °C did not lead to further changes in the intracellular tubulin levels (Figure 1B). Previous studies with another cofactor D mutant allele, *alp1-1315*, demonstrated that at 36 °C there were no changes in the level of β -tubulin, while the level of α -tubulin decreased only by 8 h of incubation (Radcliffe *et al.*, 1999). In contrast, only 10% of the *alp1-1315* cells remain viable 6 h after the shift to 36 °C (Figure 2E). We therefore concluded that there are no direct correlations between the intracellular levels of tubulin gene products and severity of

the defective phenotypes in cells with cofactor D mutations at 36 °C, so the reduced level of tubulin is unlikely to be the cause of their lethality.

***Sz. pombe* cells with defective cofactor D lose their viability during second mitosis after the G₂ phase synchronization**

The above assays showed no significant changes in tubulin levels in cofactor D mutants at 36 °C, but they did not provide information about the activity of tubulin heterodimers in these cells. We therefore turned to the analysis of cellular defects that are caused by this condition. Since *alp1-1315* allele leads to slightly more severe phenotypes at 36 °C, we used this strain for the majority of our experiments described below. We reasoned that if this mutation disrupts cofactor D function more strongly than the *alp1-t1* mutation, this might lead to a faster inactivation of tubulin heterodimers in this strain, even though tubulin levels were unchanged. To test this idea, we examined whether *alp1-1315* cells would fail in the first mitotic division at 36 °C after synchronization in G₂ phase, as opposed to *alp1-t1* cells, which divide once normally under these conditions.

Cells with *alp1-1315* mutation were synchronized in G₂ phase by centrifugation in a lactose gradient (see Materials and methods). Two cycles of cell division were analysed (Figure 2A–C). The first mitosis (100–120 min after the shift) proceeded almost normally, as judged by the low frequency of cells with abnormal cell plate index (CPI) and cytokinesis. At 220–240 min after the temperature shift, we observed accumulation of cells arrested in mitosis with hypercondensed chromosomes, which is indicative of the failure in microtubule function. Cell septation occurred a little later in mutant cells than in wild-type, suggesting that it was delayed by a mitotic checkpoint (Figure 2B, C). However, the chromosome segregation during this second division was clearly abnormal, and consequently there was an accumulation of cells with various mitotic defects (Figure 2A, panels e–i, and Figure 2C). Therefore, *alp1-1315* demonstrates the cell cycle phenotype that is very similar to that of the *alp1-t1* allele, which can also proceed through one visibly normal mitosis after the G₂ phase synchronization (Grishchuk *et al.*, 1998). The fact that the two alleles, which were isolated independently in different genetic screens and have many phenotypic differences, show similar behaviour in this test strongly suggests that the delayed onset of the microtubule defects in these strains does not mirror the kinetics of inactivation of cofactor D. Since, in mammalian cells, the cofactor D protein has other functions in addition to its role in tubulin folding (Martin *et al.*, 2000; Cunningham and Kahn, 2008), we went on to examine a possibility that fission yeast Alp1p had some essential function in G₁ or S phases of the cell cycle, thereby leading to the observed behaviour of these mutant strains after the G₂ phase synchronization.

***Alp1-1315* mutant cells lose their viability with kinetics similar to the lethality seen in cells with a mutation in the *cdc10⁺* gene, which is required to traverse the ‘start’ in early G₁ phase**

To test the above hypothesis that Alp1p may have some functions in G₁ or S phases, we calculated the transition point for *alp1-1315* using the data in Figure 2D and the procedure described in Novak and Mitchison (1989) and Nurse *et al.* (1976). The transition or execution point of a cell cycle mutation is defined as the last point in the cycle at which a

shift from permissive to restrictive temperature brings about the arrest of the ongoing cell cycle (Hartwell 1974a, 1974b; Symchen, 1978). The transition point for *alp1-1315* was -0.2 , with an error margin of 0.1 (mean $\pm$ SEM), suggesting that Alp1p function may be required from late G₂ to early G₁ phases of the cell cycle. To narrow down this range, we compared the kinetics of the loss of viability of *alp1-1315* cells with similar data for cells with mutation in the *cdc10⁺* gene. *cdc10⁺* encodes a component of the transcriptional factor that is required for traverse of the ‘start’ control in early G₁ phase and commitment to the mitotic cell cycle (Simanis and Nurse, 1989; Marks *et al.*, 1992). Mutations in the *cdc10⁺* gene lead to cell arrest before ‘start’ in G₁ phase (Simanis and Nurse, 1989). We analysed the viability of *cdc10-129* and *alp1-1315* single mutant cells at 36 °C and found that they lose viability with a similar kinetics (Figure 2E). The same results were obtained with *cdc10-M17*, another mutant allele of *cdc10⁺* (Figure 2E). Furthermore, the double mutant strain with *cdc10-129* or *cdc10-M17* and *alp1-1315* mutations also showed highly similar kinetics (Figure 2E, for *cdc10-M17 alp1-1315*; data not shown). These results were consistent with the idea that the Alp1p function was required at the same cell cycle time as for Cdc10p.

After G₁ phase synchronization, the *alp1-1315* cells proceed through S-phase normally but fail to segregate chromosomes during the following mitosis

If cofactor D performs its function in G₁ or S phases, *alp1-1315* cells synchronized in early G₁ should die in the first mitosis after they are placed at 36 °C. To test this prediction, we arrested *alp1-1315* mutant cells by nitrogen starvation at 25 °C, as described in Materials and methods, and then transferred them to 36 °C in a complete medium. Chromosome replication in *alp1-1315* was similar to wild-type cells: it started ~ 2 h after the temperature shift and the DNA content doubled by 4 h, as found with FACScan analysis (Figure 3A, B). Based on these results, it appears that progression through the S phase does not require cofactor D function. However, viability in the culture of *alp1-1315* cells began to drop soon after the temperature shift (Figure 3C, D). Analysis of *alp1-1315* cells, which were fixed and stained with DAPI and Calcofluor to visualize DNA and septa, respectively, revealed that the first mitosis occurred ~ 5 h after the shift, at which time there was an accumulation of cells with abnormal nuclear division (Figure 3E, F). The DNA content, as revealed by FACScan, was distributed broadly (Figure 3A, B).

Therefore, *alp1-1315* cells die in the first mitosis after the G₁ phase synchronization, although they can divide normally once after the G₂ phase synchronization. Similar behaviour was described for mutants in genes whose products have essential function in G₁ phase, e.g. *mis6-306* and *mis4-242* (Furuya *et al.*, 1998; Saitoh *et al.*, 1997). Therefore, the above results were consistent with the notion that function of Alp1p was required during the G₁ phase, and that failure to execute this function led to unequal chromosome segregation in the following mitosis.

Cells with *alp1-1315* mutation begin to lose their viability after 3 h at 36 °C, regardless of the cell cycle stage when the temperature was raised

Our synchronization procedures described above yielded different results depending on when in the cell cycle the cells were shifted to 36 °C. However, in both cases, after the synchronization in G₁ or G₂ phases, the *alp1-1315* cells began to lose their viability 4–5 h

after the temperature shift. After the G₂ synchronization, this corresponds to the second mitotic division, while the G₁ cells recover from the nitrogen starvation slowly and, by 4–5 hours at 36 °C, they arrive only at their first mitosis (Figure 3E, F). This observation suggested an alternative explanation to the above results. Perhaps Alp1p has no specific function during the G₁ phase, and it may even become inactivated relatively fast after the cells are shifted at 36 °C in both *alp1^{ts}* strains. However, if tubulin dimers, which have assembled at 25 °C, remain stable and functional for 2–4 h, the mutant cells might have sufficient time to complete one normal division, but they die after 4–5 h, when the tubulin dimers become depleted. To test whether the time of death for *alp1-1315* cells is independent of their passage through G₁ phase, we synchronized these cells in the G₂ phase as described above, but shifted them to 36 °C after variable delays. Samples of these cultures collected at different times after the shift to 36 °C were then fixed, the cells were stained with DAPI and Calcofluor and their phenotypes examined as described above. When synchronized cells were incubated at 25 °C continuously, all cells divided normally and the peak of cytokinesis occurred 2 h after the beginning of incubation (Figure 4A). Cells that were incubated at 25 °C for some time before shifting to 36 °C showed normal division if it occurred within 2 h after the temperature shift, regardless of the time of their incubation at 25 °C (Figure 4B). However, all cell divisions that took place after 2–3 h at 36 °C were abnormal (Figure 4C). For example, cells that were shifted to 36 °C after 60 or 120 min at 25 °C exhibited virtually identical behaviour, even though at the time of shift most of the cells in these cultures were before or after the G₁ stage. These results strongly suggest that cells with mutant cofactor D lose their viability when they attempt mitotic division with a reduced or non-functional pool of tubulin dimers, regardless of whether they passed the preceding G₁ phase at 25 °C or 36 °C.

MTs in *alp1-1315* cells become significantly impaired after 3 h of incubation at 36 °C, independently of the progression through the cell cycle

To further examine the above conclusion, we sought experimental conditions in which we could study MT cytoskeleton in cofactor D mutant cells that do not divide. We achieved this by arresting both cofactor D mutants, *alp1-1315* and *alp1-t1*, in G₂ phase at 36 °C with a mutation in the *cdc25⁺* gene. *Cdc25⁺* encodes a mitotic-phase activator, so cells with the *cdc25-22* mutation are blocked in the late G₂ phase (reviewed in Forsburg and Nurse, 1991). If Alp1p contributes to dimers assembly only during a specific cell-cycle stage, e.g. during the G₁-phase, the cytoplasmic MTs should remain stable at 36 °C in these double mutants. We examined the MT cytoskeleton in these strains by fixing cell in methanol and visualizing MTs with anti-tubulin antibodies, followed by fluorescently labelled secondary antibodies. In both *alp1-1315* and *cdc25-22 alp1-1315* cells during the first 1–2 h at 36 °C MT cytoskeleton remained normal: there were 4–8 MTs running along the cell axis and some of these MTs reached the cell ends (Figure 5A–C). However, soon afterwards the MTs began to disintegrate quickly, and by 3 h all of these cells had virtually no normal MTs. About 50% of cells contained few MTs that were short and the remaining half of the cells had no detectable MTs. Strikingly, further incubation at 36 °C did not change this result significantly, and even after 10 h of incubation up to 40% of cells still had some residual MT structures (data not shown). In contrast, in cells with *cdc25-22* mutation alone, the

cytoplasmic MTs were unchanged and remained long for at least 8 h (Figure 5A; Sawin and Nurse, 1998).

Similar results were obtained with another cofactor D mutant allele, *alp1-t1*. Initially, the MT structures in these cells were slightly reduced in number and length. Following the incubation at 36 °C, the changes in MT cytoskeleton occurred more slowly in these cells than in *alp1-1315* (Figure 5D, E). This is in a good agreement with our previous report that *alp1-t1* is partially defective at permissive temperature, but has weaker temperature sensitivity than *alp1-1315* (Fedyanina *et al.*, 2006; Grishchuk *et al.*, 1998). The reduction in MT cytoskeleton in both *cdc25-22 alp1-t1* and *cdc25-22 alp1-1315* cells occurred with almost the same kinetics as in *alp1-t1* and *alp1-1315* alone, strongly suggesting that presence of the *cdc25-22* allele did not significantly affect the described phenotypes. We noticed, however, that in cells containing any of the two cofactor D mutations in the background of the *cdc25-22* mutation, MTs were retained slightly longer than in the analogous cultures but with wild-type *cdc25⁺* gene. This might have resulted from the fact that the *cdc25-22* mutation increases the cell length even at 25 °C, so in this genetic background the MTs are initially longer than in the cells with the cofactor D mutations alone. Furthermore, the *cdc25-22* mutation prevents these cells from entering mitosis at 36 °C, and thus they avoid the complete dispersion of their cytoplasmic MTs, which normally occurs during preparation for mitosis.

After 5 h of incubation at 36 °C, both double mutant strains, *cdc25-22 alp1-t1* and *cdc25-22 alp1-1315*, began to lose their normal morphology (Figure 5F). Formation of the cell branches is a known consequence of the disruption of cytoplasmic MTs (Sawin and Nurse, 1998). Based on these observations, we concluded that MT structures in cells with mutations in cofactor D become dispersed after 2–4 h at 36 °C, even when their progression through the cell cycle is blocked.

Discussion

Tubulin folding cofactor D is a ubiquitous protein that plays a prominent role in the formation and maintenance of the MT cytoskeleton (Lopez-Fanarraga *et al.*, 2001; Szymanski 2002). It accomplishes this function in part by promoting the assembly of α - and β -tubulins into functional heterodimers (Tian *et al.*, 1996, 1997), but it may also contribute by limiting the life-span of already folded dimers (Martin *et al.*, 2000). In addition, cofactor D in *Sz. pombe* can regulate MT cytoskeleton as a MT-associated protein (Hirata *et al.*, 1998). In HeLa cells, it is also required for the recruitment of γ -tubulin ring complex to the centrosome (Cunningham and Kahn, 2008). Its removal by siRNA in these cells leads to multipolar spindle formation, but cytoplasmic MTs are not affected (Cunningham and Kahn, 2008). Thus, cofactor D is a conserved and important component of cytoskeletal organization, but how these various functions are regulated and whether they are different in different cell types is not well understood.

Our previous work has suggested that cofactor D in fission yeast may have a specific function that is required in the G₁ and/or S phases of the cell cycle (Grishchuk *et al.*, 1998). In this work we examined this hypothesis to find a definitive answer. Here we show that,

although the level of mutant cofactor D decreases when the cells are grown at restrictive temperature, the level of α - and β -tubulins does not change significantly for up to 5 h incubation at 36 °C. Therefore, cofactor D does not regulate tubulin levels in fission yeast significantly, similarly to human cofactor D (Cunningham and Kahn, 2008). Importantly, the detectable changes in tubulin levels in mutant cells of two different cofactor D alleles occur after the cells have already undergone the catastrophic division. We take this as an indication that the time of loss of cell viability in these cells does not mirror exactly the intracellular level of cofactor D and/or its activity.

We then pursued a hypothesis that the timing of cell death in cofactor D mutants is dictated by its function during a specific cell cycle stage. Some of our results with synchronized cell cultures strongly suggested that cofactor D had an execution point in G₁ phase. Indeed, *alp1-1315* mutant cells failed in the first or second mitotic division after the cells were synchronized in either G₁ or G₂ phases and then shifted to the restrictive temperature (Figures 2C, 3). Such a phenotype is sometimes indicative of a vital function of the corresponding gene product in G₁ phase of the cell cycle (Furuya *et al.*, 1998; Saitoh *et al.*, 1997). However, we obtained conclusive evidence that cofactor D in fission yeast does not have such function, and that the above cell cycle phenotype is likely to result from a decrease in concentration of active tubulin heterodimers after 2–4 h of incubation at the restrictive temperature. This conclusion is supported by our findings that synchronized *alp1*^{ts} cells lose their viability after about 3 h at 36 °C, regardless of the cell cycle stage at which they were shifted to this temperature (Figure 4B). Furthermore, when progression through the cell cycle is blocked by the *cdc25-22* mutation, the cytoplasmic MTs in cells with mutant cofactor D remain visibly normal for only about 2 h at 36 °C, but then MTs disintegrate quickly (Figure 5).

Thus, it is likely that the decrease in cofactor D activity in mutant cells results in the depletion of functional tubulin dimers, thereby affecting the length and abundance of cytoplasmic MTs. Strikingly, individual MT fragments can exist in these cells for a long time (>10 h); in asynchronous cell cultures by this time virtually all cells die due to mitotic failure (Figure 5). This result is consistent with a recent finding that the removal of human cofactor D by siRNA from HeLa cells disturbs the spindle formation to a greater extent than the cytoplasmic MTs (Cunningham and Kahn, 2008). The prolonged existence of cytoplasmic MT fragments in mutant fission yeast cells at restrictive temperature might be caused by a residual activity of mutant cofactor D. However, this seems unlikely because this phenotype is observed in two cofactor D mutants with different degree of temperature sensitivity. The presence of residual cytoplasmic MTs in cofactor D mutants could be due to the activity of other cofactors. This possibility also seems unlikely, because cofactor D plays a predominant role in the assembly of tubulin dimers in *Sz. pombe*: its absence cannot be compensated for by overexpression of any other cofactor, while the overexpression of cofactor D compensates single deletion of essential cofactors B and E (Radcliffe *et al.*, 1999). Thus, we favour the hypothesis that, even in the absence of active tubulin-folding pathway, there is some level of formation of tubulin dimers, which can polymerize into MTs. Some aspect of these structures, however, precludes formation of the functional mitotic spindle, so in the absence of the cofactor-mediated formation of tubulin dimers, the cells are unable to segregate their chromosomes normally, most likely because of the disrupted

kinetochore–MT attachments (Fedyanina *et al.*, 2006; Grishchuk *et al.*, 1998; Hirata *et al.*, 1998).

Within the frame of this hypothesis, our results allow rough estimation of the life-span of functional tubulin dimers *in vivo* upon the loss of cofactor D function. MT structures disintegrate abruptly ~3 h after incubation at 36 °C (Figure 5B), so there appears to be an active mechanism that limits the life-span of tubulin dimers to only this period, which is approximately equal to the time it takes fission yeast cells to undergo a full cell cycle. Interestingly, this time is several-fold smaller than the estimated dissociation time of bovine dimers *in vitro* (Caplow and Fee, 2002). Tubulins are highly conserved proteins (Little and Seehaus, 1988), which suggests that the life-spans of tubulin dimers in different organisms should be similar. Our finding therefore implies that the life-span of tubulin dimers might match with the duration of cell cycle, because the cell cycle in mammalian cells is significantly longer than in fission yeast (~16 h vs. 2 h). A future task will be to identify factors that regulate the life-span of tubulin dimers. This function may be carried out by tubulin cofactors other than cofactor D, which have the dimer-splitting activity and participate in the process of sorting and refolding of damaged dimers (Kortazar *et al.*, 2006, 2007; Martin *et al.*, 2000).

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Figure 1.

Protein levels of Alp1-t1p and α 1-, α 2- and β -tubulins in asynchronous cultures of *alp1-t1* mutant cells. Western blot analysis of levels of Alp1-t1p (A) and α -, β -tubulins (B) in asynchronous cultures in *alp1-t1* (SP 26) and wild-type (SPW 20) cells incubated at 36 °C for the times indicated (see Materials and methods). The plots represent average data from two independent experiments or gel loadings. Error bars here and all other graphs are standard error of the mean (SEM). The relative levels of Alp1-t1 and α -, β -tubulins were measured relative to glucose 6-phosphate dehydrogenase (G6PD) and Cdc2p

Figure 2.

Kinetics of the phenotypic changes in synchronized and asynchronous cultures of *alp1-1315* cells at restrictive temperature. (A–C) Wild-type (SPW 20) and *alp1-1315* (SPM 112) cells were incubated at 25 °C in YES and shifted to 36 °C (time zero) after synchronization in early G₂ phase. (A) Schematics of observed phenotypes: a, b, cells with condensed chromosomes; c, in anaphase; d, normal cell plate index (CPI) — cells with normally positioned septum and equal chromosome masses; e, f, g, h, abnormal CPI — cells with unequally divided chromosomes and usually normal-looking septum; i, abnormal cytokinesis — separation of daughter cells with unequally divided chromosomes. The plots for wild-type (B) and *alp1-1315* (C) cells show data for one of three independent experiments with similar results. (D, E) Log-phase cell cultures of *cdc10-129* (SPM 158), *alp1-1315* (SPM 112), *cdc10-M17* (SPM 176) grown in YES at 25 °C were transferred to 36 °C (time zero); samples were taken every 30 min and the cells were plated on YES-containing agar. Cell concentration (D) and viability (E) were analysed in at least two independent experiments

Figure 3.

Progress through the cell cycle of wild-type and *alp1-1315* cells at 36 °C after G₁ phase synchronization. Wild-type (SPW 2) and *alp1-1315* (McI 468) were arrested in G₁ by nitrogen starvation at 25 °C in synthetic medium and then transferred to YES medium at 36 °C (zero point). (A, B) DNA contents determined by FACScan. Control is a log-phase wild-type culture (SPW 20). Cell concentration (C) and viability (D). (E, F) Cytology of wild-type (E) and *alp1-1315* (F) cultures. The remaining cells in these cultures were in interphase. For each point, at least 300 cells were analysed in four independent experiments

Figure 4.

Analysis of cell division in *alp1-1315* cells synchronized in G₂ phase and incubated at 25 °C for the times indicated before shifting to 36 °C. *Alp1-1315* (SPM 112) cells were synchronized in early G₂ phase, as described in Materials and methods, incubated at 25 °C (A) in YES or shifted to 36 °C (B, C) at the times indicated. Septation and cytokinesis were analysed in at least two independent experiments. For each point, at least 300 cells were analysed

Figure 5.

Cytoplasmic MTs in cofactor D mutant cells during their arrest in G₂ phase with the help of *cdc25-22* mutation. Log phase cell cultures of *cdc25-22* (SPM 173), *alp1-1315* (SPM 112), *alp1-t1* (SP 26), *cdc25-22 alp1-1315* (SPM 154), *cdc25-22 alp1-t1* (SP 105) were grown in YES at 25 °C, then transferred to 36 °C (zero point). (A) MT structures after 4 h at 36 °C in *cdc25-22* (a), *cdc25-22 alp1-1315* (b) and *cdc25-22 alp1-t1* (c). (B–E) The plots represent the number of cells with 4–8 normal or slightly shorter cytoplasmic MTs (normal), cells with 3–4 slightly shorter MTs (reduced slightly), cells with significantly shorter MTs (reduced), and cells without any tubulin staining (absent). (B) Data for *alp1-1315* cells, except for solid triangles, which correspond to *cdc25-22*. (F) Percentage of branching cells in *cdc25-22*, *cdc25-22 alp1-1315* and *cdc25-22 alp1-t1* at 36 °C. For each point on plots (B–F), at least 300 cells were analysed in at least two independent experiments

Table 1*Sz. pombe* strains used in this study

Strain	Genotype	Source
SPW 2	972 <i>h</i> ⁻	Laboratory collection
SPW 20	<i>leu1-32 ura4-D18 h</i> ⁻	Laboratory collection
SP 26	<i>alp1-t1 leu1-32 ura4-D18 h</i> ⁻	Grishchuk <i>et al.</i> , 1998
SP 69	<i>alp1-t1::gfp::(pk1)₃::ura4⁺ leu1-32 ura4-D18 h</i> ⁻	This study
McI 468	<i>alp1-1315 h</i> ⁻	This study
SPM 112	<i>alp1-1315 leu1-32 ura4-D18 h</i> ⁻	Hirata <i>et al.</i> , 1998
SPM 173	<i>cdc25-22 leu1-32 ura4-D18 h</i> ⁻	Ogden and Fantes, 1986
SP 105	<i>cdc25-22 alp1-t1 leu1-32 ura4-D18 h</i> ⁻	This study
SPM 154	<i>cdc25-22 alp1-1315 leu1-32 ura4-D18 h</i> ⁻	This study
SPM 158	<i>cdc10-129 leu1-32 ura4-D18 h</i> ⁻	Matsumoto <i>et al.</i> , 1987
SPM 176 s	<i>cdc10-M17 leu1-32 ura4-D18 h</i> ⁺	Verkade and O'Connell, 1998