Tubulin folding cofactor D is a microtubule destabilizing protein

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Abstract A rapid switch between growth and shrinkage at microtubule ends is fundamental for many cellular processes. The main structural components of microtubules, the αβ-tubulin heterodimers, are generated through a complex folding process where GTP hydrolysis [Fontalba et al. (1993) J. Cell Sci. 106, 627–632] and a series of molecular chaperones are required [Sternlicht et al. (1993) Proc. Natl. Acad. Sci. USA 90, 9422–9426; Campo et al. (1994) FEBS Lett. 353, 162–166; Lewis et al. (1996) J. Cell Biol. 132, 1–4; Lewis et al. (1997) Trends Cell Biol. 7, 479–484; Tian et al. (1997) J. Cell Biol. 138, 821–823]. Although the participation of the cofactor proteins along the tubulin folding route has been well established in vitro, there is also evidence that these protein cofactors might contribute to diverse microtubule processes in vivo [Schwahn et al. (1998) Nature Genet. 19, 327–332; Hirata et al. (1998) EMBO J. 17, 658–666; Fanarraga et al. (1999) Cell Motil. Cytoskel. 43, 243–254]. Microtubule dynamics, crucial during mitosis, cellular motility and intracellular transport processes, are known to be regulated by at least four known microtubule-destabilizing proteins. OP18/Stathmin and XKCM1 are microtubule catastrophe-inducing factors operating through different mechanisms [Waters and Salmon (1996) Curr. Biol. 6, 361–363; McNally (1999) Curr. Biol. 9, R274–R276]. Here we show that the tubulin folding cofactor D, although it does not co-polymerize with microtubules either in vivo or in vitro, modulates microtubule dynamics by sequestering β-tubulin from GTP-bound αβ-heterodimers.

Key words: Molecular chaperone; Cytoskeleton; Microtubule dynamic; Tubulin folding cofactor

1. Introduction

Microtubules are ubiquitous and essential cytoskeletal structures assembled from heterodimers of α- and β-tubulin polypeptides. The enormous versatility of microtubules depends critically on their dynamic instability properties which are regulated by a balance between stabilizing and destabilizing factors. In living cells, most microtubules exchange subunits with a soluble tubulin pool switching abruptly between states of elongation and rapid shortening. These kinetics are governed in part by the hydrolysis of GTP bound to β-tubulin [9]. Both, the GTP hydrolysis coupled to tubulin incorporation into microtubules and the size of the tubulin-GTP cap determine microtubule dynamics. The fact that this dynamic turnover is slower in vitro than in vivo suggests the existence of additional proteins that could modulate microtubule dynamics in vivo.

The correct folding of α- and β-tubulin polypeptides and the generation of functional αβ-heterodimers require the assistance of the cytosolic chaperonin CCT and a series of additional molecular chaperones named cofactors A–E [1–5]. Various in vivo findings suggest that these tubulin folding cofactors could play additional roles in microtubule dependent processes [6–8]. For example, the *Schizosaccharomyces pombe* homologue of cofactor D (CoD), Alp1, colocalizes with microtubules and leads to reduced cell viability and microtubular abnormalities when overexpressed or deleted, respectively [7].

2. Materials and methods

2.1. CoD gene cloning, cell transfection and immunofluorescence

A total of 3 μg of DNA containing the bovine CoD gene cloned in the PCDNA3 vector (Invitrogen) and purified through a Nucleobond x500 cartridge (Nybro Macherey-Nagel) were employed for each transfection experiment. These were performed in the presence of Lipofectamine Plus (Gibco BRL) as described by the manufacturer. Cells were grown for the times indicated in the text before fixation with 4% paraformaldehyde. Immunofluorescence began with a blocking step in a buffer containing 0.1% Triton X-100, 5% FCS in PBS. Cultures were then incubated for 30–45 min at 37°C with anti-α- or anti-β-tubulin antibodies (Amersham) and the polyclonal anti-CoD antibody (kindly provided by Dr. N. Cowan). The secondary antibodies used were goat-anti-rabbit FITC and goat-anti-mouse Cy^3 (all from Jackson Immunoresearch Laboratories). Cell nuclei were stained with Hoechst 33258 (0.05 μg/ml). Finally, processed specimens were mounted in a glycerol based medium with antifading and were photographed directly.

2.2. In vitro transcription and translation of CoD and β3-tubulin

Full length CoD cDNAs cloned into PCDNA3 and β3-tubulin cloned into pGEM-2 vector were used as templates for coupled transcription and translation [10] in rabbit reticulocyte cell-free lysates (Promega Corporation) in the presence of [^{35}S]methionine (Amersham Pharmacia Biotech) for 45 min at 30°C.

2.3. CoD/tubulin co-polymerization

A total of 50 μl of purified brain tubulin was allowed to polymerize in the presence of 50 μl of the above radioactive CoD reaction product, 1 mM GTP and glycerol (30%) for 30 min at 37°C. After a first centrifugation step where the first pellet (P1) and supernatant (S1) were obtained, a second polymerization cycle was performed with the tubulin obtained in P1 after depolymerization on ice for 45 min. The second pellet (P2) and supernatant (S2) were obtained after a second centrifugation step.

3. Results and discussion

Recent in vitro studies have shown that, apart from folding tubulin, CoD can capture β-tubulin in CoD/β-tubulin complexes by disrupting native αβ-heterodimers [3,4]. Based on these results, we reasoned that an increase of CoD levels

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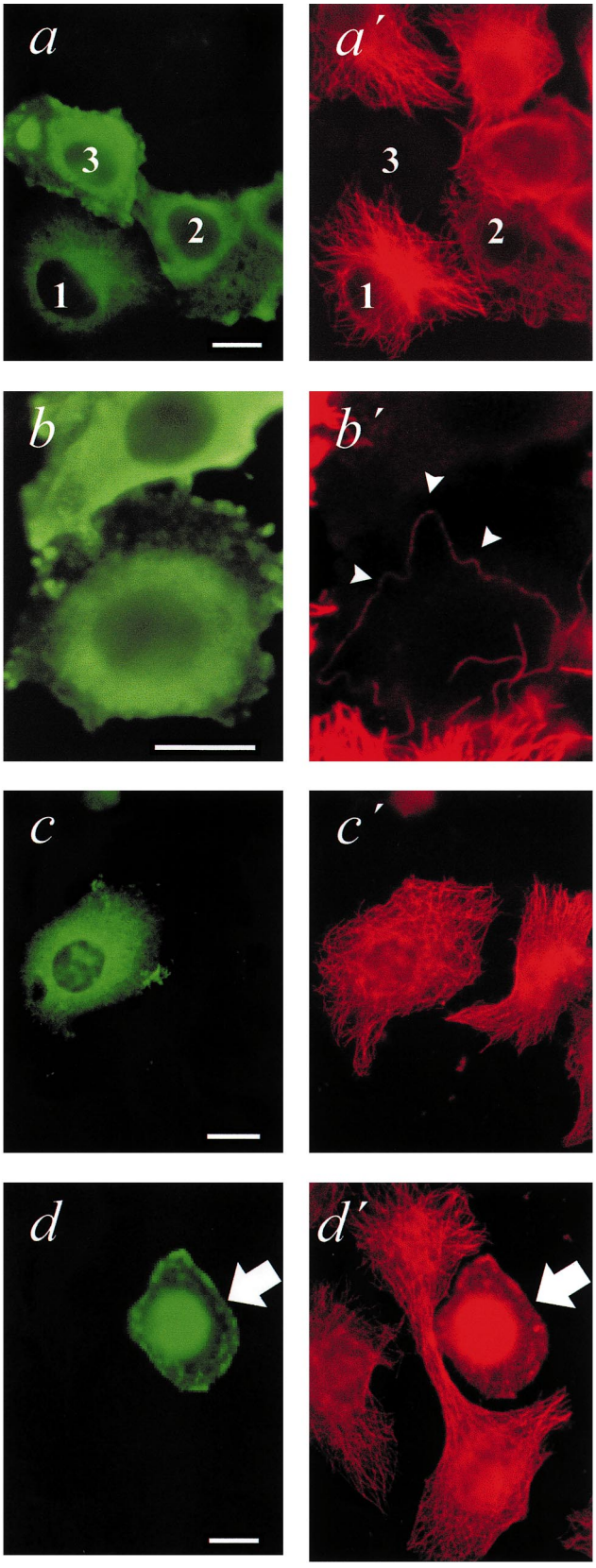

Fig. 1. CoD overexpression in mammalian cells. Double immunostaining against CoD (a–d) and α- (a′, b′) or β-tubulin (c′, d′) in CoD overexpressing HeLa cells reveals that excess of CoD correlates with microtubule depolymerization. (a′) While some CoD overexpressing cells still have an apparently normal microtubule cytoskeleton (a′, 1, c′), others present a severely reduced microtubule network (a′, 2) to an extent where no microtubules (a, 3), or a unique entire microtubule (b′, arrowheads) are detected. Cells which presented severely reduced microtubule numbers displayed a cytoplasmic form of β-tubulin (d′, arrow) which appeared to colocalize with CoD. A cytoplasmic form of α-tubulin was not detected. Scale bar represents 10 μm.
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would provide information on its function and on its possible role in microtubule dynamics. In order to investigate the in vivo effect of CoD overexpression, we transfected HeLa and NIH3T3 cells with a construct bearing the human CoD gene under control of the strong CMV promoter. Immunofluorescence labelling of these cultures with either anti-α- or anti-β-tubulin antibodies revealed a progressive loss of microtubules in CoD overexpressing HeLa (Fig. 1) or NIH3T3 (data not shown) cells, leading to a rapid drop of α-tubulin levels (Fig. 1, panels a′ and b′) but not of cytoplasmic β-tubulin immunostaining (Fig. 1, panels c′ and d′). Interestingly, treatment of transfected cultures with the protease inhibitor ALLP (Calpain-proteasome inhibitor, Calbiochem) resulted in a rise of cytoplasmic α-tubulin immunostaining and thus suggested that free α-tubulin subunits could disappear from the cell via proteasome degradation [11] (data not shown). The β-tubulin subunit on the other hand, seemed to colocalize with CoD throughout the cell (Fig. 1, panels d and d′) hence indicating that as seen in vitro, overexpressed CoD had sequestered β-tubulin monomers into CoD/β-tubulin complexes. To better understand the correlation between the overexpression of CoD and the disappearance of microtubules, we compared the amount of microtubules in CoD overexpressing cells versus controls on the same coverslips at 24, 32 and 42 h after transfection. These cell counts revealed that after 24 h one third (30%) of the CoD overexpressing cells already had no detectable microtubules. By 42 h after transfection 93% of CoD overexpressing cells were found to contain a severely reduced amount of microtubules, where approximately 57% of CoD overexpressing cells had no observable microtubules. This progressive loss of microtubules was, however, not accompanied by induction of apoptosis, as observed by DNA Hoechst staining and in situ programmed cell death identification of nuclear DNA fragmentation with the tunnel technique [12] (data not shown).

Alp1 has been shown to cosediment with taxol-stabilized mammalian microtubules and to colocalize to both spindle and interphase microtubules in fission yeast [7]. The above immunostaining experiments however, showed no CoD associated to microtubules. In order to confirm this result, we next performed a series of co-polymerization experiments employing brain tubulin [13] and partially purified in vitro synthesized radiolabeled CoD [14]. The results of these experiments shown in Fig. 2 revealed that after two co-polymerization cycles CoD did not cosediment with tubulin (P_2) and thus demonstrate that CoD does not bind to microtubules in vitro hence supporting the above in vivo observations.

Microtubule binding differences between CoD and Alp1 might reflect the absence of the CLIP-170 microtubule-binding repeat on CoD and its presence on *S. pombe* Alp1. This domain was found in human CoE, but was absent in Alp21 (its fission yeast homologue). Since CoD/β-tubulin associates with CoE/α-tubulin and CoC into an intermediate supercom-

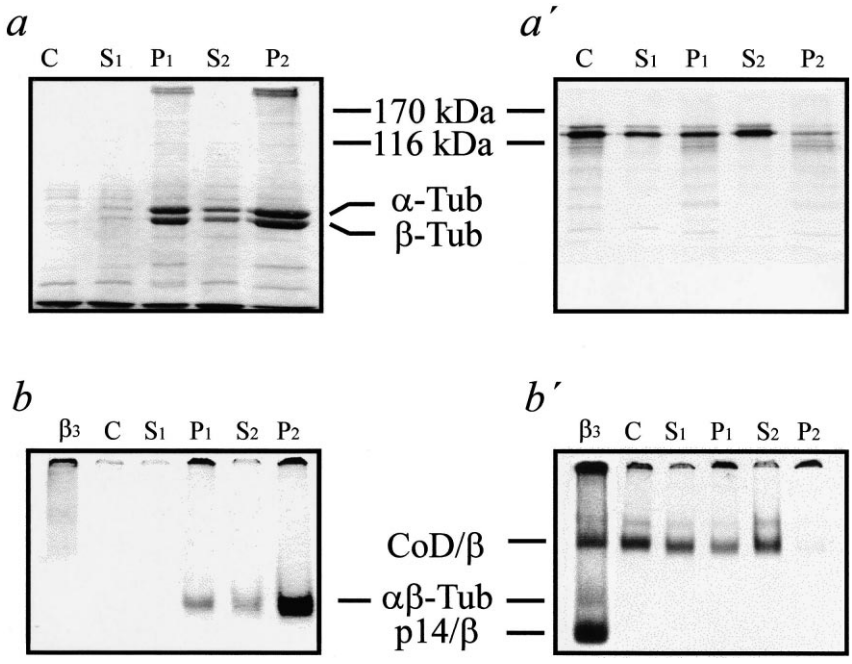

Fig. 2. CoD does not co-polymerize with microtubules. (a) Coomassie blue SDS–PAGE gel and its fluorography (a′) obtained after two brain tubulin/radioactive CoD co-polymerization cycles. Radioactive CoD is detectable in the control (C) (in vitro transcription/translation reaction) and the supernatants resulting after the two polymerization cycles (S1, S2). CoD is however not detectable in the second pellet (P2), thus indicating that CoD does not co-polymerize with microtubules under these conditions. (b) Coomassie blue-stained gel and its fluorography (b′) where the same aliquots as above have been run under non-denaturing conditions. In vitro synthesized β3-tubulin [14] has also been run as a marker for the CoD/β-tubulin complex, αβ-heterodimer and p14/β-tubulin monomer bands.

plex for the final release of the native tubulin heterodimer [4,5], it could be hypothesized that the CoD/E/C complex would interact with the microtubule through the CoE, CoC or both subunits instead of CoD. Additionally, our studies (recently supported by Tian et al. [15]) have shown that CoD has a much lower affinity for GDP-β-tubulin than for GTP-β-tubulin (data not shown) and that cofactor D/β-tubulin complexes form very inefficiently from GDP-tubulin. This suggests that CoD would preferably interact with GTP-bound β-tubulin disrupting tubulin dimers, rather than GDP-tubulin incorporated in the microtubule. Though cofactor D was found throughout the cell and did not colocalize with microtubules in vivo or in vitro, we can not rule out the possibility that cofactor D would also bind to microtubule ends through its selective binding to GTP-bound β-tubulin present in the GTP cap. In either case, catastrophe could result in the removal of the tubulin-GTP cap. Tubulin cofactors D/E/C, recently shown to behave as GTPase-activating proteins in vitro [15], might function as Stathmin which has been recently proposed to operate by modulating β-tubulin binding to GTP [16,17].

Summarizing, the above results conclude that CoD is a microtubule destabilizing protein in vivo which would increase microtubule catastrophe, either directly or indirectly, by capturing GTP-bound β-tubulin in situations where this phenomenon is required by the cell.

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