Role of 300 kDa complexes as intermediates in tubulin folding and dimerization: characterization of a 25 kDa cytosolic protein involved in the GTP-dependent release of monomeric tubulin

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β-Tubulin synthesized *in vitro* in rabbit reticulocyte lysate is found associated with 900 kDa complexes (C900) containing T Complex Polypeptide 1 (TCP1), heat-shock protein (hsp) 70 and other unidentified proteins, with smaller 300 kDa complexes (C300) of unknown nature, in dimeric association with reticulocyte α-tubulin and in monomeric forms. Pulse–chase experiments indicated that production of fully functional β-tubulin was preceded by its association with C900 and C300 multimolecular complexes and by the appearance of β-monomers. The high-molecular-mass forms appeared as intermediate products in the process leading to fully functional dimerizable β-tubulin. C300-associated tubulin can be released as β-monomer by addition of a cofactor present in reticulocyte lysate. Here a 25 kDa protein which releases tubulin monomers from C300 has been identified and characterized. The protein specifically released monomers from C300, but not from C900, in a process favoured by GTP.

INTRODUCTION

The folding *in vivo* of many proteins is not a spontaneous process. Nascent polypeptides associate co-translationally with chaperone molecules of the heat-shock protein 70 (hsp 70) family, which may stabilize them in a partially folded state, as well as protect them from premature aggregation (for reviews see [1,2]). In *Escherichia coli*, the partially folded polypeptides bind to DnaK, the *E. coli* homologue of hsp 70, and then complete their folding only after they are transferred to GroEL, the heat-shock protein 60 (hsp 60) [3]. In eukaryotic cells, sequential interaction of first hsp 70 and then hsp 60 chaperonin has been demonstrated for proteins that fold after import into mitochondria [4] and is also likely to occur in chloroplasts and in the bacterial cytosol. Little is known about the folding assistance given to cytoplasmic proteins. T Complex Polypeptide 1 (TCP1) constitutes the first cytosolic chaperonin homologue to the *E. coli* GroEL and mitochondrial hsp 60 identified so far [5–8]. Several denatured proteins, which include actin, tubulin and luciferase, are refolded *in vitro* in the presence of purified cytoplasmic TCP1 complexes [5,6] in a manner similar to the previously described folding process *in vitro* accomplished by the GroEL/GroES proteins of *E. coli* [9,10]. TCP1 complexes are hetero-oligomeric structures of 900 kDa with ATPase activity, containing two hsp 70 molecules and about six other unidentified proteins; under the electron microscope, like GroEL, they appear as a double ring structure [5–7]. So far, however, no equivalent to the GroES of *E. coli* has been identified in the cytoplasm of eukaryotic cells.

When α- and β-tubulin are synthesized in rabbit reticulocyte lysate, the newly translated polypeptides associate with a 900 kDa complex containing TCP1 (C900) [8,11]. In addition, the newly synthesized tubulin is also found associated with 150–300 kDa molecular complexes (C300) [11–13]; the nature of these complexes is not entirely known, although there is evidence that they represent intermediate stabilized forms of the folding process [8,11,13]. I show here that the newly synthesized β-tubulin enters the C900 and later the C300 complexes, before the appearance of monomeric or dimeric forms. Furthermore, I have characterized a 25 kDa protein (p25) responsible for the release of tubulin monomers from these complexes in a process favoured by GTP.

MATERIALS AND METHODS

Synthesis *in vitro* of β-tubulin and fractionation of tubulin-associated C900 and C300

Full-length cDNA encoding the mouse β-3 tubulin isotype cloned into Sp6-5 vector (Promega) was transcribed *in vitro* and translated in a micrococcal-nuclease-treated rabbit reticulocyte system (Promega), in the presence of [^{35}S]methionine (Amersham; ⩾ 1000 Ci/mmol) for 1 h at 30 °C. Translation products (100 μl) were centrifuged at 100000 *g* for 30 min at 4 °C, and applied to a Superose 6 column (Pharmacia) run at 0.4 ml/min in buffer A (50 mM Mes, pH 6.7, 1 mM $MgCl_2$, 1 mM EGTA, 75 mM NaCl and 0.2 mM GTP). Fractions (0.2 ml) were collected and radioactivity was measured by using Ready caps (Beckman). Fractions containing C900 and C300, eluted at 11.2 ml and 13.4 ml respectively, were either used immediately or stored at −70 °C in 25% glycerol.

Pulse–chase experiments

Transcription *in vitro* and translation of linearized plasmids containing the mouse β-3 tubulin gene were performed as described above, except that 1 mM unlabelled methionine was added at 8 min of translation. The reaction mixture was further incubated at 30 °C, and samples obtained at the indicated times, were added with $\frac{1}{5}$ vol. of 25% sucrose in 50 mM Mes, pH 6.7, and immediately frozen on solid CO_2. Reaction products were analysed by PAGE in native 4.5% and 7% gels, as described [11]. The bands corresponding to C900, C300, dimers, monomers, and to tubulin that did not enter the gel, were excised from the

Abbreviations used: hsp, heat-shock protein; C300, 300 kDa multimolecular complexes; C900, 900 kDa multimolecular complexes; ATP[S], adenosine 5′-[γ-thio]triphosphate; GTP[S], guanosine 5′-[γ-thio]triphosphate.

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gel, solubilized in scintillation fluid, and counted for radioactivity. The values for each band are expressed as percentages of total radioactivity (C900 + C300 + dimers + monomers + radioactivity in the wells).

Tubulin monomer-release assay

Samples of reticulocyte proteins were mixed with 4 μl of radioactive tubulin–C300 complexes in a 20 μl reaction mixture containing 50 mM Mes, pH 6.7, 100 mM NaCl, 1 mM GTP and 1 mM $MgCl_2$,. and incubated for 30 min at 30 °C. Analysis by native PAGE was performed as above. Gels were fluorographed, dried and exposed to film. Bands corresponding to free monomeric tubulin and C300 complexes were excised and counted for radioactivity, and monomer-release activity is expressed as percentage of monomers relative to total radioactivity (monomers + C300).

Purification of monomer-release activity

Samples of rabbit reticulocyte lysate (2.1×10^6 μg of protein) were centrifuged at 100000 *g* for 30 min at 4 °C, then adjusted to 50% saturation with a solution of 100%-satd. $(NH_4)_2SO_4$ in 50 mM Mes, pH 6.7, stirred for 30 min at 4 °C and then centrifuged as above. Saturated $(NH_4)_2SO_4$ was added to the supernatant to a final concentration of 75% satn., and stirred and centrifuged as above. The resulting pellet was resuspended in a minimum volume of buffer B (50 mM Mes, 1 mM dithiothreitol) and dialysed for 4 h at 4 °C in the same buffer. Dialysed material (1 ml) was applied to a 5 ml P11 phosphocellulose column (Whatman) equilibrated in buffer B. Material eluted in the void volume was applied to a 5 ml DE52 DEAE-cellulose column (Whatman) equilibrated in buffer B. After a first wash with buffer B containing 50 mM NaCl, a 70 ml linear gradient of 50–550 mM NaCl was applied. Fractions containing monomer-release activity, eluted at 250–300 mM NaCl, were concentrated and desalted by using Centricon-10 concentrators and applied to a Superdex 75 column (Pharmacia) equilibrated in buffer B. Portions (12 μl) from the fractions (0.2 ml) eluted from the column were assayed for monomer-release activity. Active fractions were concentrated by using Centricon-10 filters and stored at −70 °C in buffer B supplemented with 25% glycerol.

Other methods

Nucleotides were purchased from Boehringer Mannheim. SDS/PAGE was carried out in 14% gels by published procedures [14]. Protein concentration was determined by the method of Bradford [15], with BSA as a standard. Protein concentration in the last purification step of p25 was estimated from silver-stained SDS/polyacrylamide gels, assuming that p25 was stained with approximately equal intensity compared with the molecular-mass standards.

RESULTS

β-Tubulin associated with C300 is a precursor for dimerization-competent molecules

When examined by native gel electrophoresis, the β-tubulin translated *in vitro* is found associated with complexes of 900 and 150–300 kDa, as well as in dimeric and monomeric forms [11–13]. Pulse–chase experiments consisting of an 8 min translation reaction (pulse), followed by a chase over a period of up to 45 min, indicated that the incorporation of newly synthesized β-tubulin into dimers is preceded by the appearance of the C900, C300 and monomer forms (Figure 1) [8]. Just before the chase (8 min of translation), most of the labelled tubulin (approx. 60%) appears associated with very large complexes which do not enter the gel (Table 1, chase time 0). At that point, only 20% of the tubulin is found associated with C900 and 10% with C300. Presumably most of the labelled material at the origin corresponds to newly synthesized protein in association with ribosomes and other large particles and complexes. Shortly after the beginning of the chase, the amount of the labelled tubulin that enters the gel increases and is resolved into the various complexes and conformational forms, the appearance of which follows characteristic kinetics (Figure 1 and Table 1). As the newly synthesized tubulin leaves the particulate fraction and very large complexes, it rapidly associates with C900 (Table 1, chase time 3 min; Figure 1). This is followed by a slower and steady decline of the association of tubulin with C900, which is paralleled by an increase in its association with C300 and the concomitant appearance of monomeric tubulin. By 15–22 min of chase, roughly equal amounts of tubulin are found in these three forms. While association with C900 continues to decline, the forms

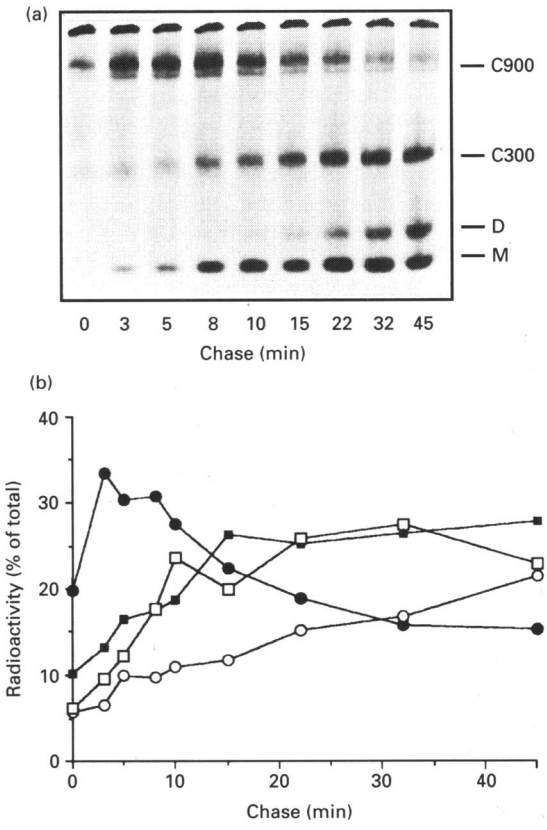

Figure 1 Pulse–chase analysis of newly translated β-tubulin by native gel electrophoresis

(**a**) Autoradiogram of non-denaturing PAGE (4.5% gel) analysis of the kinetics of the appearance of the various molecular forms of β-tubulin after synthesis *in vitro*. β-Tubulin synthesized *in vitro* was labelled with an 8 min pulse and chased for 0–45 min by addition of unlabelled methionine, with samples taken at the indicated times. In each lane 3 μl of the reaction mixture was loaded. C900, C300, M and D correspond to tubulin in the following forms: 900 kDa complexes, 300 kDa complexes, monomers and dimers, respectively, and were assigned by their elution positions compared with the elution pattern of molecular-mass markers by gel filtration on a Superose 12 column. (**b**) Relative radioactivity associated with C900 (●), C300 (■), dimers (○) and monomers (□), this being a graphic representation of the data shown in Table 1.

Table 1 Radioactivity associated with newly translated β-tubulin forms revealed by pulse–chase experiments

The bands corresponding to the different molecular forms of tubulin in the native gel shown in Figure 1 were excised and the radioactivity associated with each band at the different time points was counted. The values are expressed as percentages relative to total counts. Origin = radioactivity in the well.

Chase (min)	Origin	C900	C300	Dimer	Monomer
0	58.3	19.7	10.2	5.7	6.1
3	36.9	33.4	13.4	6.7	9.6
5	30.7	30.4	16.4	10.1	12.3
8	23.9	30.9	17.5	9.8	17.8
10	18.9	27.6	18.8	11.0	23.6
15	19.1	22.4	26.3	11.9	20.1
22	14.2	19.0	25.3	15.5	26.0
32	12.9	15.9	26.5	17.0	27.5
45	11.5	15.6	28.2	21.6	23.1

associated with C300 and tubulin monomers appear to reach a plateau by 20 min of chase. The dimeric form of tubulin shows a steady increase throughout the time of chase. At the end of the 45 min chase time, tubulin dimers continue to increase, whereas the monomeric form decreases (Figure 1). After 45 min, all these forms reach what appears as a steady state, which is maintained for at least 2 h (results not shown). Thus the kinetics of appearance of the various forms of newly synthesized tubulin suggest a rapid initial association with C900, followed by a slower transition towards C300-associated, monomeric and dimeric forms.

Exactly the same patterns and kinetics were seen when pulse–chase experiments were conducted in the presence of several types of protease inhibitors; moreover, exposure to different concentrations of trypsin [treated with tosylphenylalanylchloromethane ('TPCK')] of purified C900 containing newly synthesized labelled tubulin did not result in the appearance of any identifiable or discrete bands after non-denaturing gel electrophoresis (results not shown). These observations indicate that C300–tubulin is a stable complex corresponding to a specific intermediate folding state during the maturation of tubulin to dimerizable forms, and that it is not originated by proteolysis of C900.

The association of tubulin with C900 has been described [6,8,16] and is similar to the association of unfolded polypeptides with GroEL/GroES chaperonin proteins of *E. coli* [9,10]. In order to study the functional state of the newly synthesized tubulin associated with C300, partially purified C300 containing labelled β-tubulin was incubated with purified brain tubulin. No exchange of the newly synthesized tubulin into dimers was observed (results not shown). However, formation of functional dimerizable β-chains was observed when reticulocyte proteins were added (Figure 2a, lane 1). Therefore the newly synthesized tubulin associated with C300 complexes needs additional cofactor(s) from reticulocytes to interchange into preformed dimers.

Purification of a factor mediating the release of tubulin monomers from C300

It was previously shown that fully folded (dimerizable) β-tubulin could be generated from C300 complexes on addition of reticulocyte extracts [11,13]. Thus, I searched for the cytosolic factor(s)

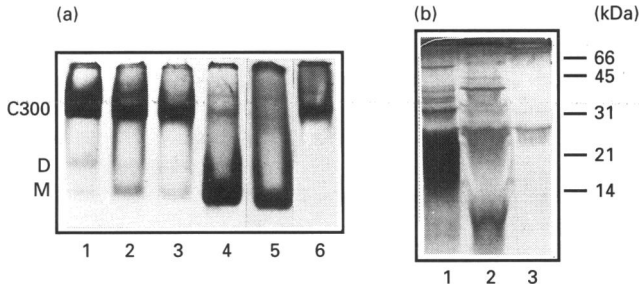

Figure 2 Purification of monomer-release activity

(**a**) Fluorography of non-denaturing PAGE (7% gel) analysis of monomer-release reactions. Purified C300 complexes containing labelled β-tubulin were incubated in monomer-release assays, with one of the following: unfractionated rabbit reticulocyte lysate (lane 1); $(NH_4)_2SO_4$ precipitate (lane 2); active fractions from P11 columns (lane 3); active fractions from DE52 ion-exchange columns (lane 4); active fractions from Superdex 75 gel-filtration columns (lane 5). Lane 6, C300 alone. (**b**) Silver-stained SDS/PAGE of samples from active fractions. lane 1, elution from P11 columns (5 μg); lane 2, DE52 active fractions (5 μg); lane 3, Superdex 75 fractions 25 and 26 (10 μl). Molecular-mass markers indicated on the right are expressed in kilodaltons. The abbreviations are the same as in Figure 1.

Table 2 Purification of C300-monomer release activity

Purification step	Total protein (μg)	Specific activity (units/μg)	Purification (fold)	Yield (%)
Reticulocytes, untreated	2.1×10^6	0.047	1	100
$(NH_4)_2SO_4$ (50–75%)	4.8×10^5	0.29	6	100
P11	4.2×10^3	10.2	217	42
DE52	252	23.2	493	6
Superdex 75	0.9	750	1040	0.6

responsible for mediating the release of β-tubulin molecules from C300. The release activity was purified in four steps, as summarized in Table 2. The native PAGE shown in Figure 2(a) represents the release activity corresponding to samples from the different steps of purification from reticulocyte lysates. Samples from untreated lysate and from the $(NH_4)_2SO_4$ precipitation steps are also included here; they were not included in the SDS/PAGE shown in Figure 2(b) due to the distortion of migration of the protein bands caused by the high concentration of globin present in reticulocytes. SDS/PAGE of the fractions from the Superdex 75 column corresponding to the peak of the release activity indicated the presence of a major band of 25.5 kDa (lane 3). The elution profile of the monomer-release activity of the last purification step, gel-filtration chromatography on a Superdex 75 column, is represented in Figure 3(a), and it appears as a single peak. A linear-regression analysis of the semi-log plot of the molecular mass versus elution position of the molecular-mass standard indicated that the protein has a molecular mass of 25 kDa, suggesting that the protein is active in monomeric form. Therefore, I shall refer to this protein as p25. In Figure 3(b), the fractions containing release activity run on SDS/PAGE indicate that the peak of activity is paralleled by an enrichment of p25.

Nucleotide requirements

Previous studies had indicated that the interchange of the newly synthesized tubulin associated with C300 into preformed hetero-

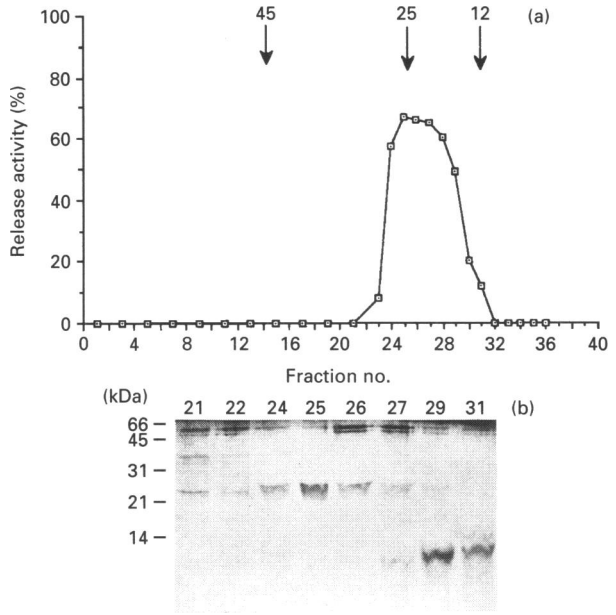

Figure 3 Enrichment of monomer-release activity by gel-filtration chromatography

(**a**) Elution profile of monomer-release activity by gel filtration on Superdex 75. Active fractions from anion-exchange columns were loaded on a Superdex 75 column, and samples from the eluted fractions were assayed for monomer-release activity by using purified labelled C300. Indicated are the elution positions of molecular-mass markers: ovalbumin (45 kDa), chymotrypsin (25 kDa) and cytochrome c (12 kDa). (**b**) Silver-stained SDS/PAGE of selected fractions eluted from the Superdex 75 column tested for monomer-release activity. The molecular sizes in kDa are shown on the left side of the panel.

Table 3 Effect of nucleotides on the monomer-release activity of p25

p25 activity was determined as described in the Materials and methods section, except that GTP in native gels and buffer was replaced by 0.05 mM GTP[S]. C300 used in these experiments was purified in buffer A without GTP.

Nucleotide	Release activity (%)
GTP	100
GDP	102
GMP	52
ATP	69
CTP	56
GTP[S]	83
GTP[S] + ATP	76
ATP[S]	25
ATP[S] + GTP	83

dimers requires hydrolysis of GTP [11]. To investigate whether this requirement applied to the release of β-tubulin monomers from C300, different nucleoside triphosphates and non-hydrolysable analogues were tested in the p25-mediated monomer-release assay. The order of preference was GTP = GDP > guanosine 5′-[γ-thio]triphosphate (GTP[S]) > ATP > CTP (Table 3). The dose/response curve for GTP indicated that the release activity increased 4.5-fold in the presence of 1 mM

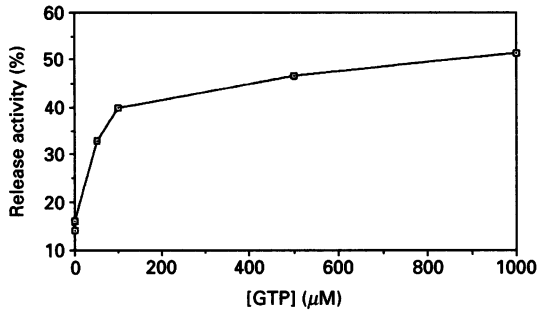

Figure 4 GTP dose–response curve for p25 monomer-release activity

The activity of p25 was assayed in the presence of variable concentrations of GTP. p25 protein (1.5 μl from concentrated Superdex 75 peak fractions) was included in a 20 μl monomer-release assay reaction mixture together with 4 μl of C300 (purified in buffer A without GTP), 2 μg of BSA, 1 mM $MgCl_2$, 100 mM NaCl and variable final concentrations of GTP. Reactions were analysed by PAGE (7% native gels), where GTP in the gel and in the buffer was replaced by 0.05 mM GTP[S]. Gels were fluorographed, dried and exposed to film. Bands corresponding to monomers and C300 were cut from the dried gel and counted for radioactivity. The release activity was measured as percentage of monomers.

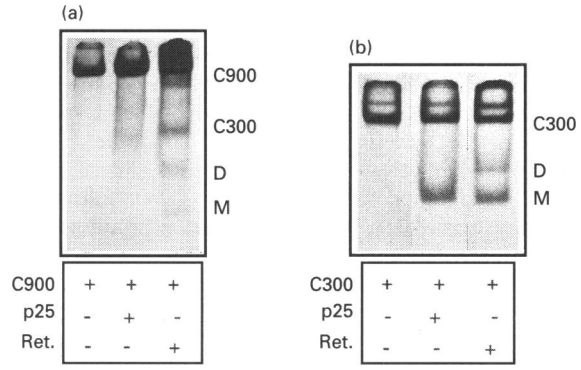

Figure 5 Substrate specificity of the monomer release activity of p25

(**a**) Monomer-release assay with purified C900 (5 μl) and p25 protein (1.5 μl) or unfractionated reticulocyte lysate (Ret.; 3 μl) was performed as described in the Materials and methods section, except that 1 mM ATP was also included in the reaction mixtures. Reaction products were analysed by PAGE (4.5% native gels). (**b**) Monomer-release assay with purified C300 and p25 protein or unfractionated reticulocyte lysate was performed as in (**a**) and analysed by PAGE (7% native gels). Abbreviations are as in Figure 1.

GTP (Figure 4). GMP and adenosine 5′-[γ-thio]triphosphate (ATP[S]) did not support the release of monomeric tubulin. When the non-hydrolysable analogue of ATP, ATP[S], was used in the presence of 0.5 mM GTP, liberation of tubulin monomers was observed, suggesting that it was the absence of GTP from the system, and not the presence of ATP[S], which prevented the liberation of monomers. Therefore the presence of GTP at micromolar concentrations significantly increases the amount of tubulin monomers released by p25 from C300. However, the release reaction does not require hydrolysis of GTP, since comparable amounts of monomers are liberated in the presence of GTP or the non-hydrolysable analogue GTP[S].

Release activity of p25 is specific for the C300 complex

Recently, two different cofactors from reticulocyte lysate have been described as necessary for refolding *in vitro* of tubulin from isolated components [16]. In particular, one of these factors

(Factor A) appears to release monomeric tubulin from the TCP1-containing 900 kDa complex. I therefore attempted to determine whether p25 was able to release tubulin monomers from such complexes. The addition of p25 to purified C900 complexes containing labelled β-tubulin (see the Materials and methods section) did not result in the appearance of any other forms of tubulin (Figure 5a). By contrast, the addition of unfractionated reticulocytes to the same complexes induced the appearance of tubulin associated with C300, tubulin heterodimers and monomers (Figure 5a, lane 3). As expected, p25 induced the release of tubulin monomers from purified C300 (Figure 5b, lane 2). When reticulocytes were added to the same complex, β-tubulin was found in dimeric and monomeric forms, indicating that additional cofactors are needed, beside p25, for the β-tubulin contained in C300 to interchange within the heterodimer (lane 3).

DISCUSSION

Newly synthesized β-tubulin polypeptides enter 900 and 300 kDa complexes before they are competent for dimerization with preformed α-tubulin. The role of C900 in the process leading to completely folded and fully functional tubulin polypeptides has been thoroughly analysed [5,6,8,16]. That the association with C300 β-subunits synthesized *de novo* is also an early event in this process is indicated by the pulse–chase experiments shown here, and suggested by previous work [8,11]. The newly synthesized β-chains associated with reticulocyte proteins in a complex of 300 kDa before appearance of the monomeric and dimeric forms, suggesting that the formation of the complex is an intermediate step in the process leading to fully folded dimerization-competent tubulin. Long-lived conformational intermediates have been observed for several other heterodimeric proteins before subunit association [17–20] and they may represent a general theme involving the post-translational association of multimeric proteins [18]. Although C900–tubulin appears to be the source of C300-associated tubulin forms, both C900– and C300–tubulin might contribute to the steep increase in monomeric forms observed in the first 5–8 min of chase (Figure 1), possibly due to the combined action of release factors liberating monomeric tubulin from C900 (Factor A) [16], and from C300 (p25). The decrease in monomeric tubulin starting at 30 min of chase time may be related to the increased proportion of dimers released from C300 and C900 complexes at this time. The kinetically slow appearance of the dimeric forms with respect to tubulin monomers suggests the occurrence of a rate-limiting step at the dimer-formation stage [21], possibly linked to the requirement for GTP hydrolysis [11]. The ratio between the different forms of tubulin stabilizes at 45 min and does not change at least over the following 2 h of incubation. This might be a reflection of the decrease, under a certain threshold, in available α-tubulin as dimerization proceeds, since the reaction can be driven forward by adding purified brain tubulin, which results in increased yields of labelled dimers and a parallel decrease in labelled C300 [11].

The nature of the C300 is not known. Indirect evidence would suggest that they might represent smaller forms of C900–chaperonin complexes. The refolding of tubulin *in vitro* accomplished by purified C900 leads to dimerizable tubulin upon addition of reticulocyte factors, but also to what appears as C300–tubulin [16]; in addition, C300 and C900 complexes were found to be co-eluted on anion-exchange on Mono Q (R. Paciucci, unpublished work). However, a proteolytic origin of these complexes from C900 seems to be unlikely, since pulse–chase experiments performed in the presence of a variety of protease inhibitors display a pattern of bands identical with that shown in Figure 1, and treatment of purified C900 with trypsin fails to give rise to complexes of 300 kDa.

The observation that, in addition to β-tubulin, α- and γ-tubulin, hsp 70 and elongation factor 1α are found associated in multimolecular complexes of molecular mass close to 300 kDa in nocodazole-growth-arrested Chinese-hamster ovary cells [22], might imply the involvement of such complexes in specific microtubule dynamic events other than folding assistance. Furthermore, these types of protein–protein complexes involving the newly translated β-tubulin polypeptides might also serve a variety of other regulatory functions [23].

The β-tubulin associated with C300 was not able to interchange with native tubulin heterodimers (R. Paciucci, unpublished work), although it did so in the presence of additional cytosolic cofactors (Figures 2a and 5b). A decreased competence for assembly of high-molecular-mass complex-associated tubulin has been previously reported [12]. Thus it would appear that the C300-associated tubulin is in a 'pre-native' or incompletely folded state. Competence to interchange within preformed dimers or for assembly into microtubules would be acquired in the presence of additional specific cofactors.

In the search for cytosolic cofactors that could mediate the release of dimerization competent tubulin from C300, I have purified a 25 kDa protein (p25) which induced the release of pre-competent monomeric tubulin, but not dimers or fully competent monomers. The release was favoured by GTP, GDP and the non-hydrolysable analogue GTP[S], and did not require the hydrolysis of GTP or ATP (Table 3). The mechanism by which this release occurs is not clear: p25 might compete with β-tubulin for binding to C300, or, alternatively, it may cause a conformational change in the tubulin molecule or the complex which results in the subsequent release of the β-chain. Guanine nucleotides might be needed for tubulin to acquire this conformational change. This hypothesis would be consistent with the observations that (i) tubulin binds guanine nucleotides (GTP/GDP) preferentially [24] and (ii) increasing amounts of β-tubulin monomers are recovered in the presence of GTP/GDP [12,13]. Alternatively, guanine nucleotides might be needed in order to activate the tubulin-release activity of p25. On the other hand, the guanine-nucleotide-dependent release of tubulin from complexes may be reminiscent of the dissociation of small GTP-binding proteins from nucleotide-exchange factors when an excess of GTP is present [25].

It has been reported that refolding of denatured α- and β-tubulin dimers by TCP1 containing chaperonin purified from bovine testis can occur in the absence of other cofactors [5]. A more recent report [16] has shown that the refolding of denatured α- and β-tubulin by TCP1-containing chaperonin purified from rabbit reticulocytes does require additional cofactors to produce functional tubulin. The experiments shown here seem to agree with the latter report, since β-tubulin synthesized *in vitro* is released from C900 and C300 and found in dimerization-competent state only in the presence of specific factors. This seeming contradiction might be explained by the different experimental systems and the use of different sources of labelled tubulin probes. Thus Frydman et al. [5] report the use of ^{125}I-labelled phosphocellulose-purified brain microtubules. This method of purification may not be sufficient to exclude the presence of contaminating release factors in the reaction with TCP1–chaperonin. Also, it can be speculated that the oxidation of tubulin during iodination might introduce modifications in the protein which overcome or decrease the requirement for additional cofactors. In the study by Gao et al. [16], labelled tubulin was obtained from cDNAs expressed in bacteria. Here, I have used tubulin–C900 purified from reaction mixtures translated *in*

vitro in rabbit reticulocytes. Both preparations require additional cofactors for the formation of tubulin dimers, arguing against a possible action of contaminants from the partial purifications of the labelled probes in the folding reaction. The other component present in the refolding reactions, i.e. C900, was purified under similar conditions by Frydman et al. [5] and Gao et al. [16], and thus it is unlikely that the purification scheme or source of these complexes can account for the observed differences. Furthermore, the tubulin–C900 used in the present study, partially purified by size exclusion, still required cofactors from reticulocyte lysates for the release of dimers. Therefore C900 do not seem to be physically associated with such factors, at least with sufficient affinity to allow co-purification.

p25 has no monomer-release activity on C900 (Figure 5a). This, together with the differences in size and the behaviour on ion-exchange chromatography (R. Paciucci, unpublished work), provide evidence that p25 may be distinct from the factor recently reported [16].

Based on the above observations and on previous studies, it is proposed that the steps leading to fully folded and dimerization-competent β-tubulin proceed in the following order. (1) Nascent β-tubulin polypeptides associate with C900, where at least initial folding reactions take place [8,16]. (2) When the former reaction is completed, β-tubulin becomes associated with C300 in a process requiring cytosolic factors and ATP hydrolysis [8,11,16]. C300 might be the site for further folding reactions which no longer require association with C900, and for assistance in the dimerization with α-tubulin, perhaps by regulating the rate of hydrolysis of GTP required for the formation of tubulin heterodimers [11]. (3) Monomeric β-tubulin would then be released from C300, in a p25-mediated reaction, or directly from C900 [16]. (4) The release of heterodimers from C300 would require factors which are distinct from p25 and possibly from factors which release tubulin from C900 (R. Paciucci, unpublished work).

Monomeric β-tubulin is observed in reaction mixtures translated *in vitro* [8,12,13,21] and by immunoblots of extracts of reticulocytes, and thus constitutes a conformational state present *in vivo* [11,26]. However, free β-tubulin monomers do not interchange within preformed dimers, nor do they co-polymerize with microtubules *in vitro* [12,16], and are considered as 'conformational intermediates' or 'incompletely folded' molecules [11,16,21]. Since dimers (i.e., fully functional tubulin) can be released from C300 with the assistance of cytosolic factors distinct from p25, what would be the significance of the monomer-release reaction *in vivo*? It could be hypothesized that the release of β-tubulin monomers as 'not readily utilizable tubulin' from C300 complexes accomplished by p25 might be a mechanism controlling the rate of dimer formation *in vivo*. In the absence of p25, for example, in refolding experiments *in vitro* from isolated components, no β-monomeric forms are detected [16], suggesting that most of the input unfolded product goes to its final conformation (dimer). It could be argued that, *in vivo*, the regulation of the rate of tubulin dimerization could prevent additional dimer formation in rapidly depolymerizing microtubules. On the other hand, free β-tubulin monomers might be involved directly in the control of tubulin synthesis [23].

I thank J. Avila for helpful comments and suggestions during the preparation of the manuscript, and J. C. Zabala for providing laboratory space, reagents and equipment. I am very grateful to T. Thomson, who provided reagents and equipment, in addition to critically contributing to the design of many experiments, thoroughly reviewing the manuscript, and providing financial support during this work.

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Received 29 September 1993/2 February 1994; accepted 9 February 1994