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A 14 kDa release factor is involved in GTP-dependent β-tubulin folding

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Abstract The tubulin folding pathway is a model system to understand protein folding in the cell. It involves the interaction of several chaperones, including TCP-1 and other as yet uncharacterized factors. Release of tubulin monomers from folding intermediates (C_{900} and C_{300}) and their incorporation into tubulin dimers is dependent on GTP hydrolysis, magnesium ions and release factors. In this work, we have purified to homogeneity the protein factor responsible for the release of β-tubulin monomers from C_{300} complexes. It has an apparent molecular mass of 14 kDa (p14) as judged by SDS electrophoresis. The protein behaved as a dimer of about 28 kDa when analyzed by gel filtration chromatography. Furthermore, the p14-dependent release of β-tubulin monomers from C_{300} complexes takes place in the presence of GTP. These results suggest that p14 is a new chaperone that assists in tubulin folding by facilitating the acquisition of the native conformation.

Key words: Native electrophoresis: Tubulin folding; Release factor; Protein purification

1. Introduction

One intringuing question in biology is how proteins adopt their native conformations. Proteins are frequently observed to fold very fast, but a unique conformation of a protein cannot be encountered by random fluctuations [1]. In the cell, protein folding occurs in connection with other cellular processes including assembly from individual polypeptide chains. Protein folding has been shown to be assisted by proteins that function as molecular chaperones. A new member of the chaperonin family, TCP-1, has been identified in the eukaryotic cytoplasm. It is a 60 kDa subunit of a hetero-oligomeric chaperone involved in the folding of actins and tubulins [2–7]. Six *Tcp-1*-related genes encoding subunits of the TCP-1-containing chaperonin have been recently identified, reinforcing their role in the folding of different eukaryotic proteins [8].

In vitro synthesis of tubulins gives rise to the formation of different multimolecular complexes (termed C_{900} and C_{300}), in addition to monomers and dimers. These complexes are intermediates in the pathway of tubulin folding and dimer formation. TCP-1-containing chaperonin forms a binary complex with completely denatured tubulin [9].

This interaction appears to be a fast first step in the tubulin folding and assembly process [7]. Release of partially folded tubulin from C_{900} complexes occurs after a transition of the chaperonin to the ATP-bound state [10], in a manner analogous to the dissociation of target proteins from complexes formed with hsp70 [11]. Release of tubulin monomers from C_{300} complexes, in which TCP-1 is already absent, and their incorporation into tubulin dimers is dependent on GTP hydrolysis, magnesium ions and release factors present in cellular extracts [12]. Also, release of tubulin monomers from C_{900} complexes and their incorporation into tubulin dimers has been shown to be dependent on two unknown protein cofactors of about 40–45 kDa termed A and B [9].

To better understand the process of tubulin folding and dimer formation, we have used the monomer release assay based on non-denaturing electrophoresis of tubulins [13], to purify to homogeneity the protein factor responsible for the release of tubulin monomers from C_{300} complexes. This release factor, termed p14, is a new cytoplasmic chaperone that behaves as such, facilitating tubulin folding in a process dependent on GTP.

2. Materials and methods

2.1. Materials

Reticulocyte extracts were obtained from Promega, [^{35}S]methionine (>1,000 Ci/mmol) from Amersham and GTPγS and ATPγS from Boehringer Mannheim.

2.2. Partial purification of C_{300} β_3-tubulin complexes

cDNA encoding wild-type β_3-tubulin cloned into pGEM-2 was used as template for coupled in vitro transcription and translation [14] in a rabbit reticulocyte cell-free system in the presence of [^{35}S]methionine for 75 min at 30°C. C_{300} complexes were partially purified as described [13].

2.3. Native gel electrophoresis

Protein samples were diluted with 25% sucrose (final concentration of 5%) in 50 mM MES, pH 6.7 (loading buffer) and immediately loaded onto a 7% or 4.5% non-denaturing polyacrylamide gel [11,12] containing ME buffer (100 mM MES, pH 6.7, 1 mM EGTA) with 0.1 mM GTP and 1 mM $MgCl_2$. Following electrophoresis, gels were fluorographed, dried and exposed to film (Hyperfilm MP, Amersham).

2.4. Monomer release assay

Aliquots of protein preparations were mixed with 2 μl of labelled C_{300} complexes in 10 μl reactions containing 50 mM MES, pH 6.7, 1 mM GTP, and 1 mM $MgCl_2$, and were incubated for 30 min at 30°C. These reactions were diluted with loading buffer and loaded onto a 4.5% or 7% non-denaturing polyacrylamide gel.

2.5. Pulse–chase experiments

Purified brain tubulin was prepared as previously described [12,13]. Full-length cDNA encoding wild-type β_3-tubulin [15] cloned into pGEM-2 vector was used as template for in vitro transcription [16]. Transcribed mRNA was translated in a rabbit reticulocyte cell-free system [17] in the presence of [^{35}S]methionine for 15 or 20 min at 30°C. Cold methionine (1 mM) was added to the translation reactions. 2 μl aliquots of the reactions that contain an energy generating system present in the reticulocyte lysates (Promega) were incubated at 30°C for the times indicated in the figures and immediately loaded onto a native gel. In other experiments, the reactions were dialyzed for 5 h. against 50 mM MES pH 6.7 or diluted with the same buffer and concentrated by ultrafiltration using Microcon filters (Amicon). 2 μl aliquots of the dialyzed reactions were incubated at 30°C for the times indicated in the figures with purified brain tubulin (0.3 mg/ml), 1 mM $MgCl_2$, and

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different nucleotide combinations: 1 mM GTP, or 1 mM ATP, or 1 mM GTPγS plus 0.5 mM ATP, or 1 mM ATPγS plus 0.5 mM GTP. Aliquots from these reactions were diluted with loading buffer and immediately loaded onto a 4.5% non-denaturing polyacrylamide gel [12,13].

2.6. Protein purification

For purification of p14 release factor, monomer release activity was monitored through all purification steps by the monomer release assay using C_{300} complexes purified as described above. Pig testis (300 g) were homogenized in a commercial blender with 100 ml of ice-cold buffer A (50 mM MES, pH 6.7, 0.5 mM EGTA) containing 2 μM aprotinin, 2 μM leupeptin, 1 mM phenylmethane-sulfonylfluoride (PMSF), and 10 μM benzamidine. Glass beads were added and complete homogenization was carried out by using a Bead-Beater (Biospec) in two 1-min bursts, separated by 1 min of cooling. The homogenate was centrifuged for 45 min at 200,000 × *g* and the supernatant stored at −70°C. 20 ml aliquots were applied onto a phosphocellulose column (XK 16/20, Pharmacia) equilibrated in buffer A. The column was washed with buffer A until the A_{280} of the eluent returned to baseline. The flow-through fractions were pooled and concentrated by ultrafiltration using Centriprep-10 filters (Amicon). Aliquots of 4 ml (300 mg) were applied onto two consecutive 5 ml HiTrap blue affinity columns (Pharmacia) equilibrated in 100 mM MES, pH 6.7. The column was washed with equilibration buffer to remove unbound proteins and eluted (3 ml/min) first with a 20 ml NaCl linear gradient from 0–1 M in 100 mM MES, pH 6.7, and second with 20 ml of 1 M NaCl in 100 mM MES, pH 6.7. Monomer release activity eluted from this column at 1 M NaCl. Fractions containing monomer release activity were pooled (6 ml), concentrated and desalted by ultrafiltration by using first Centriprep-10 and second Centricon-10 filters (Amicon).

Desalted and concentrated fractions (1 ml, 20 mg) were loaded onto a Q-Resource column (6 ml, Pharmacia), equilibrated in buffer B (50 mM MES, pH 6.7; 1 mM $MgCl_2$) plus 25 mM NaCl. The column was washed at 4 ml/min with equilibration buffer until the A_{280} returned to the baseline and then eluted with a 60 ml linear gradient from 25 to 500 mM NaCl in buffer B. Monomer release activity eluted from this column at 125 mM NaCl. Fractions containing monomer release activity were pooled (3 ml) and concentrated by ultrafiltration.

Concentrated fractions from the anion-exchange column (600 μg) were applied to a high resolution gel filtration column (Superdex-75, Pharmacia) equilibrated and eluted at 0.75 ml/min with 100 mM MES, pH 6.7. The monomer release activity eluted from this column as a sharp peak in the 11.9 ml (28 kDa) and was estimated to contain p14 protein purified to apparent homogeneity (50 μg).

2.7. Peptide sequencing of p14

Purified p14 was diluted in 0.1 M ammonium bicarbonate pH 8.5, and digested with trypsin (Merck) for 12 h. at 37°C. Tryptic peptides from p14 were separated by reverse-phase HPLC in a SMART System equipped with a μRPC C2/C18 microbore column (Pharmacia). Finally, the peptide containing fractions were sequenced by automated Edman degradation on a Applied Biosystems 477A sequencer equipped with a model 120A phenylthiohydantoin analyzer.

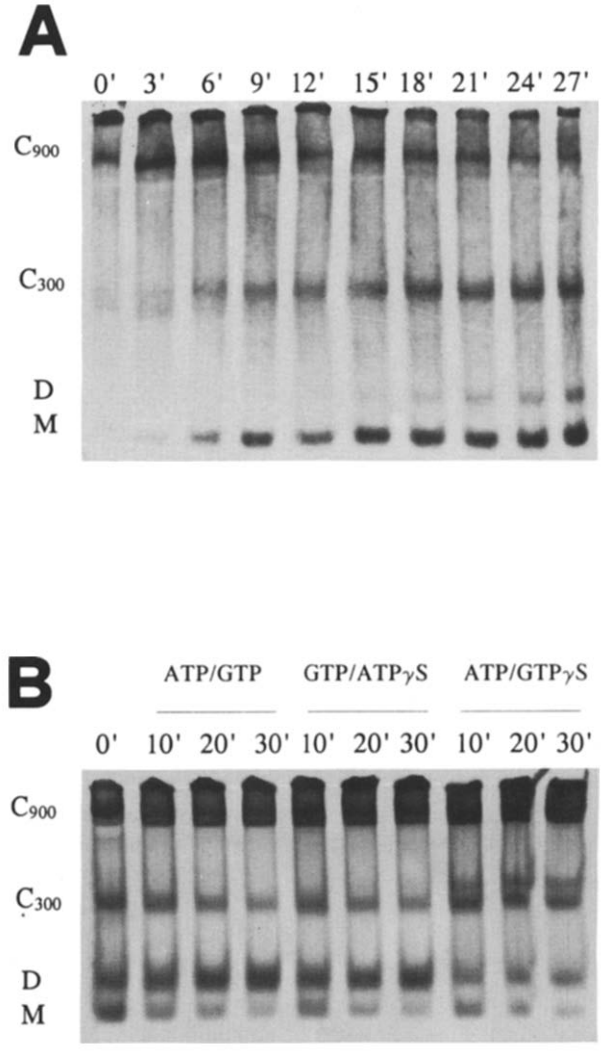

Fig. 1. Pulse–chase experiments of in vitro translated β_3-tubulin. Newly synthesized β_3-tubulin was analyzed by electrophoresis on 4.5% non-denaturing gels. Panel A shows the results of a 15 min pulse that was chased with cold methionine in the presence of nucleotides (section 2) for the times indicated. Panel B shows a similar experiment but pulse time was 20 min and chase was made in the presence of different nucleotides for times indicated. C900, C300, D, and M indicate the position of the different molecular forms appearing during in vitro translation of β-tubulins [12,13].

3. Results and discussion

Pulse–chase experiments could be used to follow the fate of newly synthesized tubulin during its folding process. Fig. 1A shows the analysis of a 15 min pulse with [^{35}S]methionine to allow complete synthesis of tubulin. Completely denatured or newly synthesized tubulin enter a TCP-1 complex to form a binary complex (C_{900}, Fig. 1A) [7,9]. These binary complexes could be separated on Superose-6 gel filtration columns where they run as a complex of about 700 kDa [7,9], and detected on 4.5% non-denaturing gels as shown with anti-TCP-1 antibodies [12]. It has been suggested that during the folding process C_{900} complexes gave rise to the formation of C_{300} complexes [7,13]. In vitro folding assays have shown that tubulin could be completely folded and incorporated into tubulin dimers when released from these complexes (C_{900} and C_{300}) in the presence of cellular factors, ATP, and GTP [7,9,12], as previously suggested [13]. Furthermore, incorporation of tubulin monomers into dimers seems to be dependent on GTP hydrolysis [12]. Though the molecular nature of C_{300} complexes remains to be established, they are probably constituted by components previously present in C_{900} complexes, since TCP-1 binary complexes give rise to the formation of completely folded tubulin in the presence of release factors.

Partially purified C_{300} complexes are capable of forming tubulin monomers and dimers in the presence of release factors and GTP [12]. Prolonged incubation of C_{900} complexes in the presence of ATP gave rise to the appearance of C_{300} complexes [7]. Pulse–chase experiments (Fig. 1A) shows that the formation of C_{300} complexes and monomers could be correlated with the disappearance of C_{900} complexes, and ultimately with the incor-

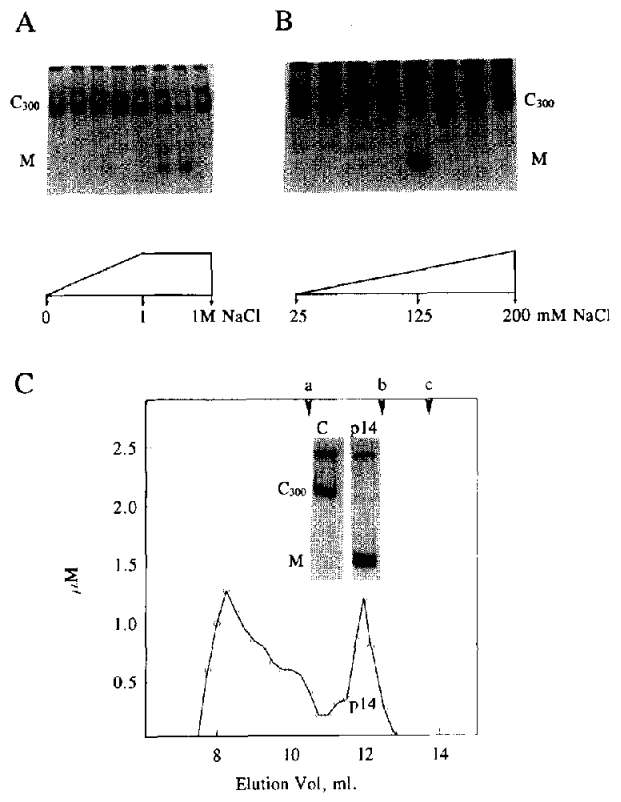

Fig. 2. Purification of p14. p14 release factor was purified from pig testis by phosphocellulose, HiTrap blue affinity, anion-exchange, and gel filtration chromatography. Fractions containing p14 protein were located by the monomer release assay (section 2), and analyzed on non-denaturing gels [12,13]. Panel A shows the analysis of fractions eluted from the HiTrap column at the salt concentrations indicated in the diagram of the figure. Panel B shows a similar analysis of fractions eluted from the Q-Resource column. Panel C shows the elution profile from the Superdex-75 HR column. Inset shows the analysis on a 7% non-denaturing gel of the monomer release activity of purified p14 protein emerging from the column at 11.9 ml (p14), and control fractions with no monomer release activity (C). a–c represent the positions of molecular size markers: a, ovoalbumin (45 kDa); b, chymotrysinogen A (25 kDa); c, cytochrome *c* (12.5 kDa).

poration of tubulin monomers into dimers. Since the release of tubulin from C_{900} complexes is dependent on ATP hydrolysis, and the incorporation into dimers from C_{300} complexes is dependent on GTP hydrolysis, we repeated the pulse–chase experiment in the presence of these nucleotides and their corresponding non-hydrolyzable analogs (Fig. 1B). Under conditions where tubulin release from C_{900} did not occur, it can be observed that the appearance of tubulin dimers originated from C_{300} complexes and/or monomers, was not precluded by ATPγS. Also, incorporation into tubulin dimers from C_{300} complexes was inhibited by the presence of the GTPγS, as previously shown [12].

A two-step mechanism has been proposed to explain how tubulin folding occurs [12]. The first step is dependent on ATP hydrolysis where the ATP/ADP exchange will direct the release of partially folded tubulin [10], in a manner analogous to the dissociation of target proteins from hsp70 [11]. In the second step, tubulin associated to C_{300} complexes will be completely folded and incorporated into tubulin dimers in the presence of GTP and release factors [12].

In order to gain a further insight into the tubulin folding process we used the monomer release assay to purify the protein factor. We have purified this release factor by using phosphocellulose, affinity, anion-exchange, and gel filtration chromatography (Fig. 2). Aliquots of each step of the purification were analyzed on 16.5% polyacrylamide gels (Fig. 3). The material contained in the p14 peak migrated as a single band with a molecular mass of 14 kDa. Fractions with monomer release activity from the Superdex column (p14) were also analyzed on polyacrylamide gels and silver stained; a single band was detected (data not shown). Thus, the monomer release activity is associated with a protein of 14 kDa (p14). p14 eluted from the Superdex-75 column in the 11.9 ml and behaved as a dimer of 28 kDa (Fig. 2).

To identify the nature of p14, a partial sequence was obtained from purified p14 protein after digestion with trypsin. Tryptic peptides were separated by reversed-phase HPLC (Fig. 4). A major peptide was found with the sequence MMIPD. This peptide sequence was compared to sequences in the PIR protein database release 39.0 and GENBANK database release 81.0 and EMBL database completed in June, 1994. No homology with any other protein sequenced so far was found.

Incorporation of unfolded tubulin into dimers has been shown to require GTP [12]. Tubulins are GTP-binding proteins [18,19]. In the absence of GTP tubulins denature very fast. Thus, it is conceivable that tubulins would bind GTP during their folding process, in which they give rise to the formation of different multimolecular complexes, monomers, and dimers. Tubulin monomers detected by non-denaturing electrophoresis seem to have GTP-bound because they are unstable if GTP is omitted in the gel and in the running buffer [13]. Also, it has been shown that tubulin monomers bind GTP exchangeably [20]. To establish the role of the GTP in the folding process at the level of monomer release directed by p14 protein, we incubated C_{300} complexes with purified p14 protein in the presence and in the absence of GTP. Fig. 5 shows that monomer release took place in the absence of added GTP, though the amount

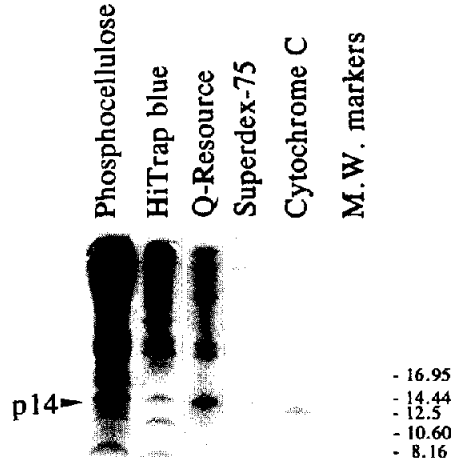

Fig. 3. Coomasie blue stained gel representing each step of p14 purification. Analysis on a 16.5% tricine-SDS-polyacrylamide gel [22] of the different steps of p14 purification. The amounts of total protein loaded in each lane are: Phosphocellulose (75 μg); HiTrap blue (15 μg); Q-Resource (10 μg); Superdex-75 HR (0.2 μg). Molecular weight of cytochrome *c* and low molecular weight standards from Sigma are indicated. Arrow shows the position of p14 protein.

of monomers detected after non-denaturing electrophoresis was remarkably lower than when the reaction was carried out in the presence of added GTP. After fluorography, the radioactive bands were excised and quantitated by scintillation counting. The amounts of monomers detected in the absence of added GTP represent the 35% of the counts associated to C_{300} complexes while they represent the 70% in the presence of added GTP. The fact that monomers were released in the absence of added GTP indicates that C_{300} complexes have GTP bound to them.

In the absence of added GTP tubulin monomers denatured fast as deduced from its low recovery and were not chased back into C_{300} complexes. Thus, GTP is also required for the stabilization of the monomeric form once released. Incubation of partially purified C_{300} complexes with GTPγS prior to the addition of purified p14 protein completely preclude the release of monomers (data not shown, manuscript in preparation). These results support the notion that GTP hydrolysis is required for monomer release as suggested by pulse–chase experiments and that p14 protein is a new chaperone implicated in the GTP-dependent monomer release step of β-tubulin folding.

During the preparation of this manuscript Gao et al. published the purification of a factor of 15 kDa from bovine testis, and the complete sequence of the corresponding mouse protein deduced from its cDNA [21]. The partial amino acid sequence here reported for p14 from pig testis matches perfectly with residues 62 to 66 present in the sequence of cofactor A from murine origin, suggesting that they are closely related or identical molecules. It is noteworthy that these authors have found that cofactor A (p14) is a cochaperonin of TCP-1 that increases its ATP hydrolysis activity, and that releases tubulin monomers from TCP-1 binary complexes. As we discussed above, C_{900} complexes are precursors of C_{300} complexes. It seems plausible that different components of C_{300} complexes would be present in C_{900} complexes though they are devoid of TCP-1 [12]. Thus, independently of its cochaperonin activity, p14 is responsible for the release of tubulin from C_{300} complexes, presumably in the absence of TCP-1. Accordingly, p14 acts during the second step of the tubulin folding pathway that has been proposed previously [12]. Though the mechanism by which p14 releases tubulin from C_{300} complexes is unclear, p14 could compete with tubulin for binding to C_{300} complexes favouring the acquisition of the folded conformation. Also, p14 itself could require GTP in order to release tubulin from C_{300} complexes, or p14 might influence the GTP hydrolysis activity of C_{300} complexes.

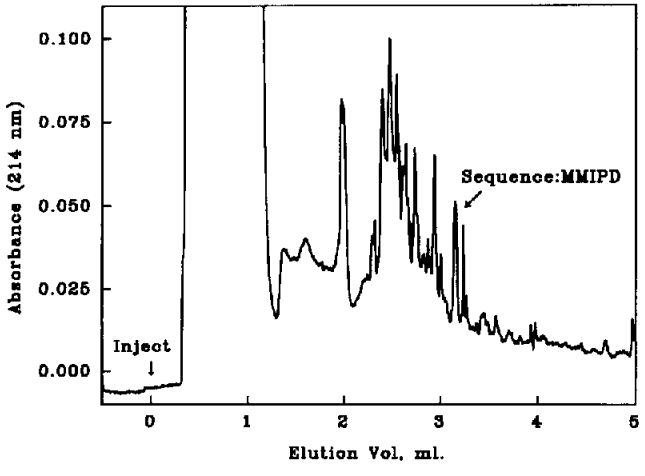

Fig. 4. Purification of tryptic peptides from p14. The tryptic digest was acidified and applied to a μRPC C2/C18 microbore column equilibrated in 0.06% (v/v) trifluoroacetic acid. The elution of the peptides was performed by a gradient of 80% (v/v) acetonitrile in 0.05% trifluoroacetic acid. The sequence obtained for the indicated peak is shown.

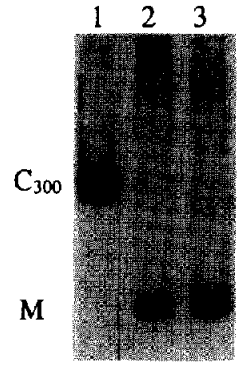

Fig. 5. Analysis of the monomer release activity of purified p14 on a 4.5% non-denaturing gel. Lane 1, partially purified C_{300} complexes incubated in buffer alone; lane 2, in the presence of p14 without GTP added; and lane 3, in the presence of p14 and 1 mM GTP. C_{300} indicate the position of C_{300} complexes and M the position of monomers.

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