In vitro formation of different tubulin polymers from purified tubulin of Ehrlich ascites tumor cells

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Preparations of cycled tubulin from Ehrlich ascites tumor cells contain several acessory proteins; once or twice cycled microtubule preparations are usually composed of fibers 10 nm in diameter, but lack vimentin. Highly purified tubulin consists of α- and β-tubulin and a minor component which was identified by peptide mapping as a second β-chain. This pure tubulin is able to form in vitro at low concentrations (1 mg protein/ml) fibers of about 10 nm width, and at higher concentrations (3.5 mg protein/ml) normal microtubules.

Microtubules *10-nm Fiber* *Ehrlich ascites tumor cell* *Second β-tubulin* *Tubulin-heterogeneity*

1. INTRODUCTION

A major recent interest in the in vitro assembly of tubulin has focused on the structure of the polymerized products and the involvement of microtubule-associated proteins or MAPs. A large number of studies have been published that define the conditions required for this reaction (for a review see [1]). In some reports it has been postulated that MAPs are absolutely necessary for the in vitro assembly of tubulin [2–4]. However, in others it has been shown that pure tubulin can readily assemble into microtubules in the absence of non-tubulin proteins [5–9]. We have previously reported that microtubules from Ehrlich ascites tumor (EAT) cells can be polymerized without the involvement of MAPs [10]. Furthermore, in a preliminary report we presented evidence for the in vitro formation of 10-nm filaments from tubulin of EAT cells [11]. Here, we show that highly pure tubulin from these cells can form in vitro, in addition to microtubules, fibers of about 10-nm diameter.

2. MATERIALS AND METHODS

Twice-cycled microtubule protein from EAT cells was prepared as in [12] as modified in [13] in buffer containing 0.1 M Pipes (piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), 1 mM $MgCl_2$ (pH 6.9). Two-dimensional gel electrophoresis was carried out as in [14]. Isoelectric focusing was done on 3% acrylamide gels containing 8 M urea and 2% ampholines (LKB) (pH 3.5–10). The anode and cathode buffers were 0.2% (v/v) sulfuric acid and 0.4% (v/v) ethanolamine. Runs were started and continued at 400 V for 14–16 h. Gels were removed, equilibrated for 30 min with a buffer containing 2% $NaDodSO_4$, 5% 2-mercaptoethanol and 0.065 M Tris–HCl (pH 6.8) and run in the second dimension on a $NaDodSO_4$ slab gel.

Vimentin from EAT cells was isolated as in [15]. Highly pure EAT-tubulin was prepared by DEAE-Sephadex chromatography as in [10]. Reduced protein samples were carboxymethylated by reaction with sodium iodoacetate [16]. The tubulin polypeptides were separated from each other by preparative gel electrophoresis. The stained bands were excised from the gel strips and were electrophoretically eluted.

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The isolated polypeptides were examined by protease digestion on 15% $NaDodSO_4$–polyacrylamide gels as in [17]. Routinely, 40–50 μg of the separated proteins were incubated at 37°C for 30 min with 0.5 μg *Staphylococcus aureus* V8 protease (36-900, Miles Laboratories, Elkhart, IN).

Samples for electron microscopy were applied to carbon-coated Formvar films cast on copper grids, stained with 2% uranylacetate and examined in a Siemens 1A electron microscope.

3. RESULTS AND DISCUSSION

Analysis by two-dimensional electrophoresis of tubulin preparations at two stages of purification by assembly/disassembly is shown in fig.1. A number of non-tubulin proteins can be seen. Most of them disappear during two cycling steps. Two spots of tubulin are visible in the cytosol preparation (fig.1a) and after one cycling step (fig.1b). α-Tubulin is seen to be less acidic than β-tubulin. A

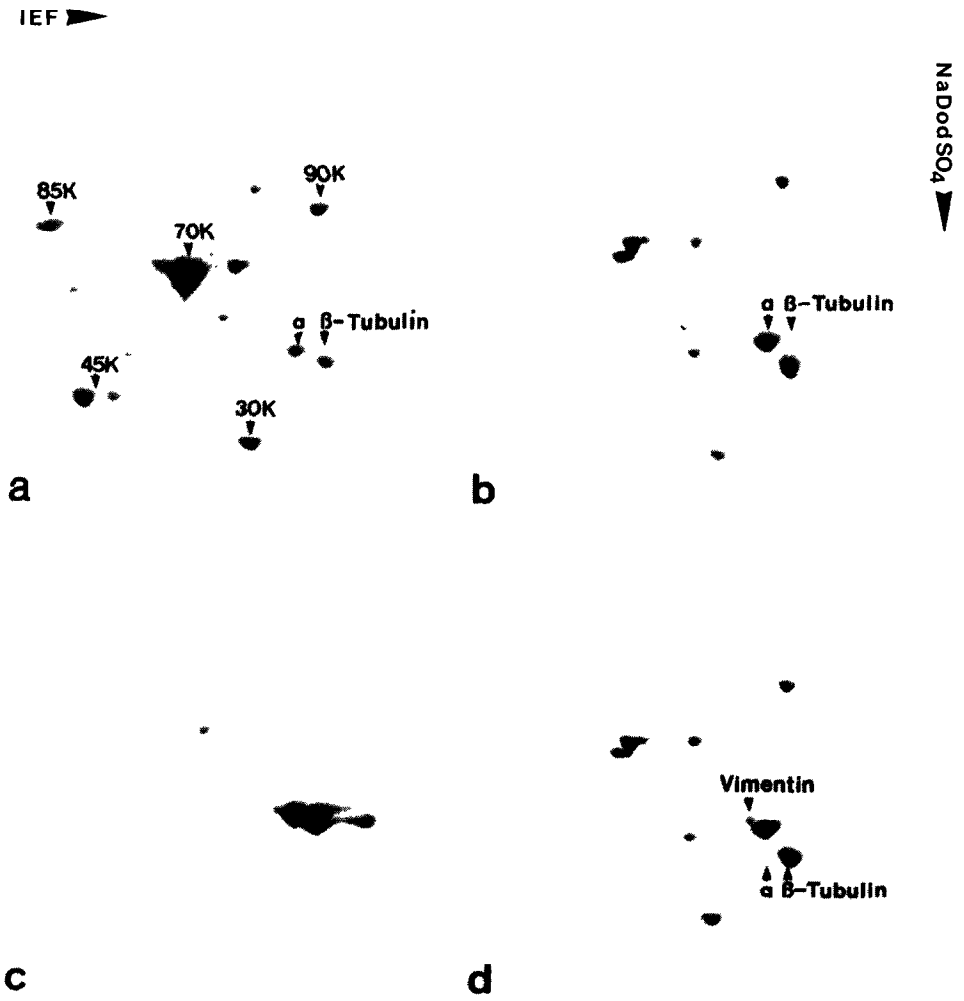

Fig.1. Analysis of fractions from EAT tubulin preparations by two-dimensional gel electrophoresis: (a) cytosol from EAT cells; (b) supernatant after the first cycling step; (c) supernatant after the second purification step; (d) mixture of (b) and 3 μg vimentin. The acidic side is on the right. The samples were loaded at the upper left (basic side) for the first isoelectric focusing (IEF) dimension. The second dimension of $NaDodSO_4$–gel electrophoresis is from top to bottom.

second cycle of assembly/disassembly dramatically improved the purity of tubulin (fig.1c), although few minor proteins and an increased tubulin heterogeneity could be observed. The nature of the tubulin heterogeneity is not known, however, it is possible that the multiple forms of tubulin are generated artifactually.

Once or twice-cycled microtubule preparations from EAT cells have usually been observed by electron microscopy to contain filaments 10 nm in diameter. To check whether these filaments were contaminations by vimentin filaments, we examined the protein composition of cycled tubulin by two-dimensional gel electrophoresis. Addition of vimentin (3 μg) from EAT cells to microtubule protein showed an additional spot located just above α-tubulin (fig.1d). This result demonstrates that the observed filaments were not derived from vimentin contamination.

In an attempt to prove that tubulin polymerizes into fibers of about 10 nm diameter and to exclude the possibility that the minor non-tubulin proteins could account for the filaments, we isolated highly pure tubulin. Fig.2a shows tubulin purified by ion-exchange chromatography on DEAE-Sephadex. There is only one band observable in the tubulin preparation. Reduction and carboxymethylation of purified tubulin revealed a minor protein on the gels, migrating between α- and β-tubulin (fig.2b). In order to identify this protein the 3 proteins were separated from each other by preparative gel electrophoresis (fig.2c–e) and were subjected to peptide mapping. The gel banding patterns of the α- and β-tubulins and the minor protein, generated by *Staphylococcus aureus* protease, are given in fig.3. The results show that the pattern of EAT α-tubulin (fig.3b) is similar to the pattern of α-tubulin from porcine brain (fig.3a). The same observation holds for the β-subunits of either source (fig.3c,d). It is notable that the pattern of the third component (fig.3e) is very similar to the pattern of β-tubulin. Thus, the minor protein is a second β-tubulin and the purified tubulin consists of nothing but 3 tubulin components. A similar se-

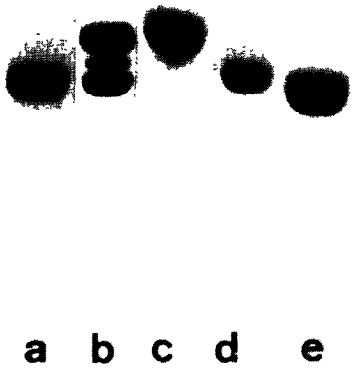

Fig.2. $NaDodSO_4$–polyacrylamide gel electrophoresis of highly purified tubulin: (a) DEAE-purified tubulin; (b) reduced and carboxymethylated tubulin; (c) α-tubulin; (d) minor protein; (e) β-tubulin.

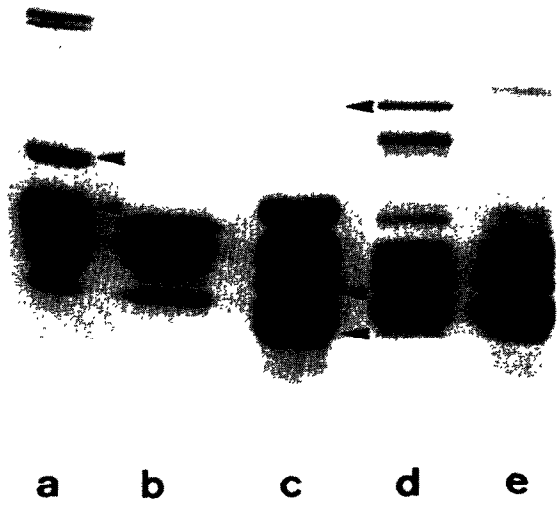

Fig.3. Characterization of the tubulin species by peptide mapping: (a) porcine brain α-tubulin; (b) EAT α-tubulin; (c) brain β-tubulin; (d) EAT β-tubulin; (e) EAT minor tubulin. The arrow heads indicate some differences in specific peptides.

cond β-chain in pig brain tubulin has already been identified [18].

Incubation of high concentrations (3.5 mg protein/ml) of purified EAT-tubulin in the presence of 1 mM GTP, at 37°C and for 20–30 min results in the formation of microtubules (fig.4a). However, when lower concentrations (1 mg protein/ml) of tubulin are incubated under the same conditions, the first detectable structures (5–10 min after incubation) are fibers of about 10 nm diameter (fig.4b). Extending the incubation time to longer than 30 min increases the amount of these filaments without observable formation of microtubules. The fibers are irregularly decorated with globular particles and are sometimes twisted. Two-dimensional gel electrophoresis of the pelleted filaments shows multiple forms of tubulin (fig.5). The observable shift in charge and in M_r-values could be due to phosphorylation and limited proteolytic modification of the tubulin polypeptides.

The studies reported here show that highly purified EAT-tubulin is able to form in vitro fibers of about 10 nm width in addition to microtubules. These 10-nm fibers are obviously distinct from those of intermediate filaments. They are formed from soluble pure EAT-tubulin. The conditions for the alternative formation of each assembly pro-

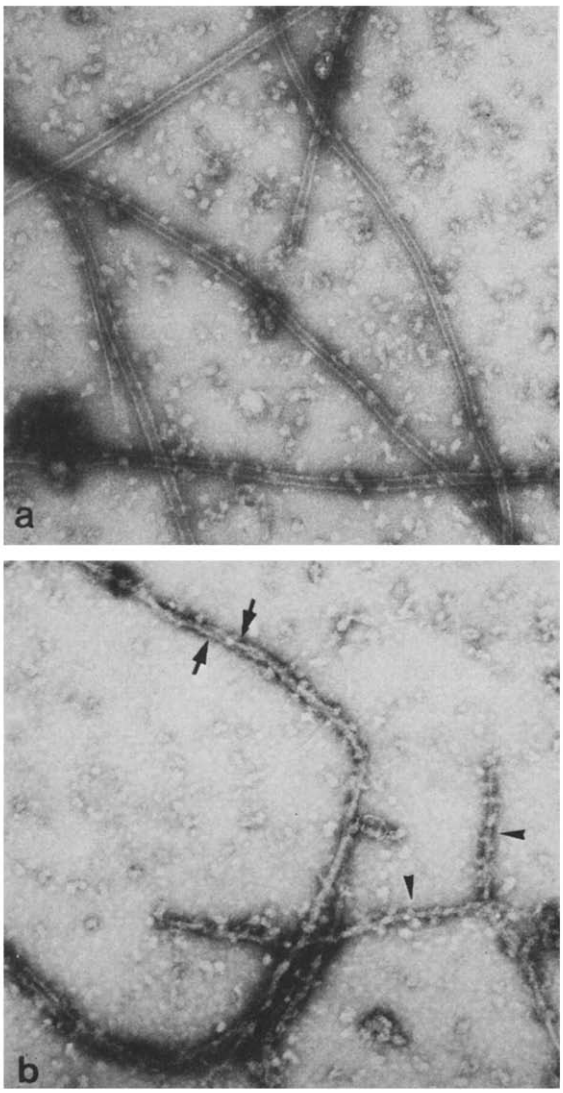

Fig.4. In vitro assembled microtubules and fibers of 10 nm width in negative stain analysis (2% uranylacetate): (a) electron micrograph of purified tubulin (3.5 mg protein/ml) after adding 1 mM GTP and warming up to 37°C for 30 min showing intact microtubules (20000 × g); (b) electron micrograph of purified tubulin (1 mg protein/ml) after addition of 1 mM GTP and elevation of the temperature to 37°C for 10 min indicating the formation of numerous filaments 10 nm in diameter (60000 × g). Arrows: twisted fibers. Arrow heads: single fibers.

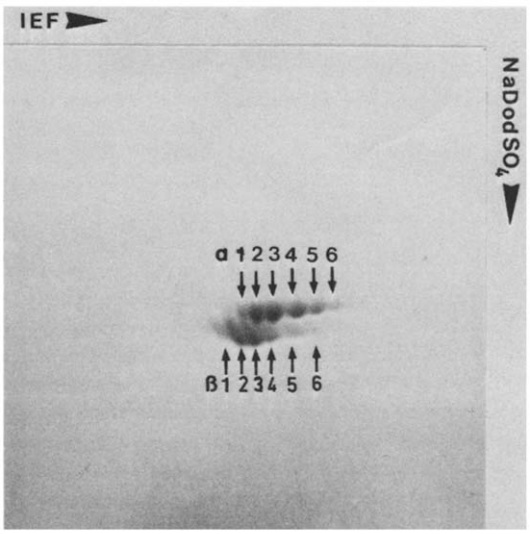

Fig.5. Two-dimensional gel electrophoresis of the assembled and pelleted fibers from highly pure tubulin. The assembled filaments were pelleted by centrifugation at 150000 × g, at 30°C for 60 min. Two-dimensional gel electrophoresis was as described in section 2. The acidic side is on the right.

duct are unclear at present. Fibers of 10 nm width were recently detected as breakdown products of the reaction of in vitro polymerized microtubules with an antibody against β-tubulin [19]. Our results show that the 10-nm fibers are formed shortly after starting of tubulin polymerization, as mentioned above, indicating the assembly from tubulin dimers. However, the presence of a protease in the tubulin preparation cannot be excluded completely. Therefore, the formation of these structures could also be explained by degradation and reassembly of breakdown products during a short time period.

In a further report it has been stated that microtubules possess semi-stable protofilaments and ribbons of protofilaments, and that these protofilament ribbons are a form of 10-nm intermediate filaments [20]. The relationship between fibers of about 10 nm diameter and intermediate filaments of the vimentin type found in EAT cells remains to be elucidated. Whether the presented results have any significance for the in vivo existence of these filaments is still difficult to answer. We are currently working to quantify the conditions for the assembly process of the described filaments, to test their sensitivity to drugs (mitotic poisons) and to look for enzymatic and other possible regulatory factors.

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