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Assembly of Nonneural Microtubules in the Absence of Glycerol and Microtubule-Associated Proteins†

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ABSTRACT: Microtubule protein from Ehrlich ascites tumor cells purified by an in vitro polymerization process in the absence of glycerol and calcium chelators contains several accessory proteins but lacks the high molecular weight proteins which are present in neurotubulin. DEAE-Sephadex chromatography of two-times cycled tubulin removes these nontubulin proteins, resulting in pure tubulin, as critically examined by sodium dodecyl sulfate gel electrophoresis. This tubulin can readily assemble into microtubules in assembly buffer, at low magnesium concentrations, without glycerol and at tubulin concentrations above 0.8 mg/mL. Electron microscopy shows that the tubules are identical with normal microtubules. When the purified tubulin fraction was reduced and carboxymethylated, a significant minor protein component could be observed electrophoretically, migrating between α- and β-tubulin. At present, the identity and function of this protein are not known. The results demonstrate that the in vitro assembly of tubulin from Ehrlich ascites tumor cells does not require high molecular weight proteins or τ-like factor(s) as has been proposed for the neurotubulin system.

To experimentally analyze the mechanisms of microtubule assembly in living cells, it is necessary to study the biochemistry of microtubules in vitro and to correlate these findings with the in vivo role of microtubules. Until recently, almost all of the information about microtubule assembly in vitro came from experiments with either partially purified tubulin or crude cytosol of mammalian brain. Microtubule-associated proteins (MAPs)[1] have been described in brain tissue that stimulate the in vitro assembly of microtubules (Gaskin et al., 1974; Dentler et al., 1975; Murphy & Borisy, 1975; Weingarten et al., 1975; Sloboda et al., 1976; Erickson, 1974; Borisy et al., 1974). These MAPs copurify with neurotubules through several cycles of polymerization and depolymerization and can be separated from tubulin by ion-exchange chromatography. The protein(s) necessary for stimulation of microtubule assembly has been designated either the high molecular weight [M_r(h)] MAPs (Murphy & Borisy, 1975; Borisy et al., 1975; Keates & Hall, 1975; Murphy et al., 1977) or the τ factor (Weingarten et al., 1975; Cleveland et al., 1977a,b). In some reports it has been postulated that MAPs are absolutely required for the in vitro assembly of neurotubulin (Weingarten et al., 1975; Sloboda et al., 1976; Bryan et al., 1975). However, in others it has been shown that purified tubulin can readily assemble into microtubules in the absence of nontubulin proteins and under appropriate conditions (Murphy et al., 1977; Lee & Timasheff, 1975, 1977; Erickson & Voter, 1976; Himes et al., 1976; Wheland et al., 1977; Herzog & Weber, 1977; Lee et al., 1978). Several investigators have described the presence of ring forms in neurotubulin complexed with MAPs (Erickson, 1974; Kirschner et al., 1974; Doenges et al., 1976; Marcum & Borisy, 1978). It has been reported that these rings may be intermediates in the assembly process and that they are probably essential for tubulin polymerization (Olmsted et al., 1974; Erickson, 1974; Kirschner et al., 1974).

Recently, the reversible in vitro polymerization of microtubules from sources other than brain has been reported (Wiche & Cole, 1976; Wiche et al., 1977; Weber et al., 1977; Doenges et al., 1977; Nagle et al., 1977; Weatherbee et al., 1978; Farrell & Wilson, 1978). It has been found that microtubule protein from nonneural and cultured cells (Doenges et al., 1977; Nagle et al., 1977; Weatherbee et al., 1978) and from sea urchin sperm tail outer doublet microtubules (Farrell & Wilson, 1978) lacks M_r(h) MAPs but contains some proteins of intermediate molecular weight associated with

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[1] Abbreviations used: EAT, Ehrlich ascites tumor; M_r(h), high molecular weight components; Pipes, 1,4-piperazinediethanesulfonic acid 5625-37-6; RB, reassembly buffer; M_r, molecular weight; MAPs, microtubule-associated proteins.

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tubulin. We have previously reported that microtubules from nonneural Ehrlich ascites tumor (EAT) cells can be polymerized by the glycerol procedure in the presence or absence of calcium chelators (Doenges, 1978) and without the involvement of M_r(h) proteins and ringlike structures (Doenges et al., 1977).

In the present study, we have used the polymerization conditions of Borisy et al. (1975) originally developed to prepare microtubules from brain extracts and have found that polymerization of tubulin from EAT cells may also occur without addition of glycerol. Furthermore, we show that in vitro microtubule assembly from these cells does not require the presence of M_r(h) or τ-like MAPs and that tubulin purified by DEAE-Sephadex chromatography is readily able to polymerize into microtubules.

Materials and Methods

Microtubule protein was prepared from EAT cells by slight modifications of the assembly–disassembly method of Borisy et al. (1975). Unless otherwise stated, all experiments were carried out in reassembly buffer (RB), containing 0.1 M Pipes, 1 mM $MgCl_2$, and 0.1 mM GTP, pH 6.9. About 150–200 g (wet weight) of EAT cell pellet was washed three times with cold RB, and the cells were resuspended in one-tenth volume of cold RB. The thick suspension was homogenized in an Ultraturrax homogenizer, and the homogenate was centrifuged at 100000*g* for 60 min at 4 °C. The crude supernatant was made 2 mM in GTP, warmed to 37 °C for 30 min, and centrifuged at 50000*g* for 30 min at 30 °C. The pellet was resuspended in one-fifth volume of the 100000*g* supernatant by homogenization in a Dounce homogenizer, cooled at 0 °C for 30 min, and centrifuged at 50000*g* for 30 min at 4 °C. GTP was added to the supernatant, and the solution was incubated at 37 °C and centrifuged at 30 °C as above. The pellet was resuspended in 5–6 mL of RB, homogenized, cooled at 0 °C, and centrifuged at 50000*g* at 4 °C. The supernatant, containing 3–5 mg of protein/mL, was frozen at −80 °C. Three-times cycled tubulin was obtained from this solution by repeating the assembly procedure, resuspending the microtubules in 1–2 mL of RB, and disassembling by cooling and centrifuging as above. The final third cold supernatant contained about 1–1.5 mg of protein/mL. When glycerol was used, the procedure of Shelanski et al. (1973) was modified as previously described (Doenges et al., 1977).

Protein concentrations were determined by the method of Lowry et al. (1951), by using bovine serum albumin as a standard. Reduced protein samples were carboxymethylated by reaction with sodium iodoacetate (Crestfield et al., 1963). Polyacrylamide–sodium dodecyl sulfate gel electrophoresis was performed on slab gels according to the method of Laemmli (1970). Phosphocellulose (Whatman P 11) chromatography of microtubule protein was carried out according to the method of Weingarten et al. (1975). Microtubule-associated proteins were separated from tubulin on DEAE-Sephadex A-50 by elution with RB and stepwise elution with KCl in RB at 4 °C. Routinely, 25–30 mg of two-times cycled microtubule protein in RB was applied to a 1 × 20 cm column and eluted with RB, RB containing 0.3 M KCl, and RB containing 0.6 M KCl. The salt-containing peak fractions were immediately desalted by dialysis against RB at 4 °C. Microtubule assembly was monitored by using the increase in light scattering at 350 nm (Gaskin et al., 1974). For electron microscopy, samples were placed for 30 s on carbon-coated Formvar films cast on copper grids and stained by rinsing with 15 drops of 2% aqueous uranyl acetate. Excess stain was removed with filter paper and the grid was air-dried.

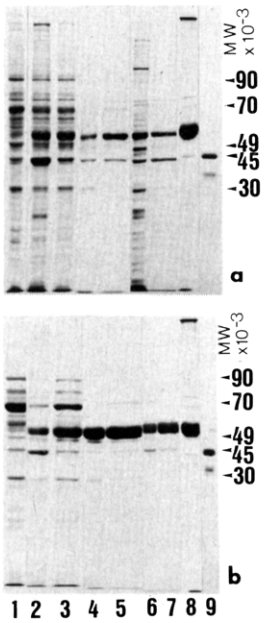

FIGURE 1: Analysis of fractions from EAT tubulin preparations by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. (a) Fractions prepared without glycerol. (b) Fractions prepared with glycerol. (1) Whole extracts; (2) pellets after first assembly; (3) supernatants after first disassembly; (4) pellets after second assembly; (5) supernatants after second disassembly; (6) pellets after third assembly; (7) supernatants after third disassembly; (8) two-times cycled brain tubulin; (9) actin, prepared from EAT cells.

Results

Assembly of Microtubules from EAT Cells in the Absence of Glycerol. In previous studies we have purified microtubule protein from EAT cells (Doenges et al., 1977; Doenges, 1978) and cultured cells (Nagle et al., 1977) by the glycerol procedure. The demonstration that tubulin from HeLa cells (Weatherbee et al., 1978) and from sea urchin sperm tail outer doublet microtubules (Farrell & Wilson, 1978) could be recycled in the absence of glycerol suggested to us that tubulin from EAT cells could also be purified without glycerol.

Incubation of highly concentrated cytosol preparations (30–40 mg of protein/mL) from EAT cells with GTP in RB at 37 °C resulted in the formation of a dense gel. After 10 min of incubation, a large number of microtubules and microfilaments could be seen by electron microscopic examination. Consequently, we subjected cytosol preparations to three polymerization–depolymerization cycles in the absence of glycerol. Microtubule formation was routinely characterized at each stage of the purification process by electrophoretic analysis on polyacrylamide–sodium dodecyl sulfate slab gels. Figure 1 shows a comparative analysis of fractions from EAT tubulin preparations in the presence and absence of glycerol. It can be seen that in both cases three successive cycles of assembly and disassembly resulted in the enrichment of tubulin. However, the amount of nontubulin proteins is greatly increased in the absence of glycerol (Figure 1a). Especially, a prominent polypeptide having a M_r of about 45 000 and migrating at the same position as EAT cell actin could be observed besides tubulin. The effect of glycerol on the composition of microtubule protein has already been described during the preparation of microtubules from mammalian brain (Scheele & Borisy, 1976) and nonneural HeLa cells (Weatherbee et al., 1978). Further minor impurities of three-times cycled tubulin are polypeptides with molecular weights of approximately 49 000, 70 000, and 90 000–110 000. No M_r(h) proteins have been observed during the cycling steps.

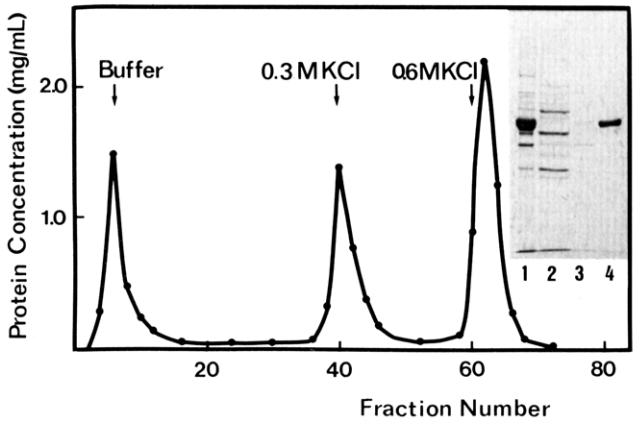

FIGURE 2: DEAE-Sephadex fractionation of two-times cycled EAT tubulin as described under Materials and Methods. Gel electrophoretic analysis of the column fractions is shown in the inset. (1) Two-times cycled EAT tubulin; (2) unbound fraction; (3) fraction eluted by 0.3 M KCl; (4) fraction eluted by 0.6 M KCl.

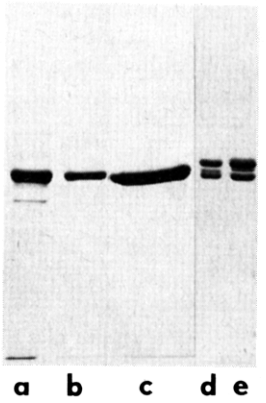

FIGURE 3: Sodium dodecyl sulfate–polyacrylamide gels of purified tubulin. (a) Two-times cycled EAT tubulin; (b) 30 μg of tubulin purified by DEAE-Sephadex; (c) 140 μg of the same sample as (b); (d) reduced and carboxymethylated tubulin from EAT cells; (e) reduced and carboxymethylated tubulin from porcine brain. The protein samples were prepared by procedures described under Materials and Methods.

In the following experiments we have used microtubule protein prepared in the absence of glycerol.

Preparation of Nontubulin Proteins and Purified Tubulin. In order to examine the role of MAPs in the assembly reaction of EAT tubulin, we attempted to remove this material by ion-exchange chromatography on phosphocellulose and DEAE-Sephadex. We found that treatment of two-times cycled EAT tubulin with phosphocellulose did not completely remove polypeptides with molecular weights of about 70 000 and 49 000 from tubulin.

Chromatography of microtubule protein on DEAE-Sephadex gave a better and cleaner separation (Figure 2). Most of the nontubulin components did not bind to the column and could be eluted by RB. A small portion of the applied sample, containing proteins with molecular weights of 90 000, 55 000, and 45 000, was eluted with 0.3 M KCl in RB. Finally, a homogeneous peak of pure tubulin was eluted with 0.6 M KCl in RB. The protein fractions obtained from this column were analyzed by sodium dodecyl sulfate gel electrophoresis as shown in the inset of Figure 2. The tubulin fractions were further subjected to stringent tests of purity by gel electrophoresis carried out at various loadings (Figure 3b,c). The results demonstrate that under protein concentrations of about 20–40 μg/gel there is only one band observable in the pure tubulin preparation. Increasing protein concentrations to 140 μg/gel showed, besides tubulin, the presence of barely detectable amounts of two components having molecular weights of 49 000 and 45 000. However, reduction and carboxymethylation of purified tubulin revealed a minor protein on the gels, migrating between α- and β-tubulin (Figure 3d). It is not yet clear whether this protein is a specific component of the microtubule system or an artifactual impurity. A similar protein was found in purified tubulin from porcine brain, although its M_r seems to differ from that found in EAT tubulin (Figure 3e).

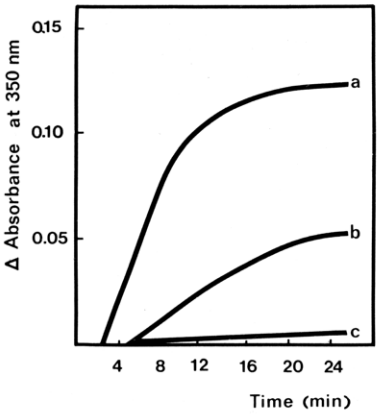

FIGURE 4: Turbidimetric measurements of purified EAT tubulin at 37 °C and 350 nm as a function of protein concentrations. Polymerization of microtubules was induced in Pipes buffer by addition of 1 mM GTP to protein solutions and elevation of the temperature from 0 to 37 °C. Protein concentrations: (a) 1.4; (b) 1.2; (c) 0.8 mg/mL.

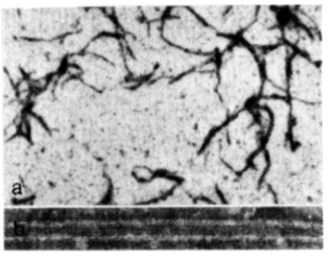

FIGURE 5: Electron micrographs of purified tubulin preparations in Pipes buffer after adding GTP and warming up to 37 °C for 20 min, showing a large number of microtubules (a) (5 000× magnification). A higher magnification (75 000×) is shown in (b). Negative staining was with 2% uranyl acetate.

Competence of DEAE-Purified Tubulin for Assembly into Microtubules. The ability of DEAE-purified tubulin to reassemble into microtubules was tested in RB solutions by turbidity experiments and electron microscopy. Addition of 1 mM GTP to the dialyzed tubulin fraction, containing 1.2 mg of protein/mL, and elevation of the temperature from 0 to 37 °C resulted in an increase of turbidity, indicating the formation of aggregates. The effect of different tubulin concentrations on the formation of aggregates is shown in Figure 4. Increasing the protein concentration shortens the lag time before the onset of aggregation and increases both the rate and extent of assembly. No increase in turbidity was detectable at protein concentrations at or below 0.8 mg/mL. From these data the critical free-protein concentration necessary for aggregation must be of the order of 0.8–1 mg/mL.

The structure of the aggregates was characterized by electron microscopy. After 15–20 min following addition of GTP to purified tubulin samples and transfer to 37 °C, the electron micrographs indicate the presence of numerous filaments having the typical appearance of microtubules (Figure 5). The diameter of the observed filaments was approximately

23 ± 3 nm, similar to the dimensions of native microtubules. The microtubules are cold labile but are more stable to calcium than neurotubules (Doenges, unpublished experiments). No ringlike structures could be observed in these preparations. The results of these turbidity and electron microscopic experiments clearly show that microtubules can be assembled from DEAE-purified EAT tubulin without the addition of any other proteins or other factors.

The protein fraction, isolated on DEAE-Sephadex by elution with 0.3 M KCl, was still able to form aggregates when incubated in RB. The presence of nonseparated tubulin in this material obviously accounts for this observation. In contrast, the protein fraction that is not adsorbed to the DEAE column did not give turbidity. Furthermore, mixing of these nontubulin proteins with purified tubulin (1.2 mg/mL) did not significantly increase the formation of microtubules.

Discussion

Our studies reported here and previously (Doenges et al., 1977) have shown that tubulin from EAT cells can be purified by two or three cycles of assembly–disassembly in the absence or presence of glycerol. It is evident that the amount of nontubulin proteins present in microtubules depends on the method of purification. Microtubule protein prepared by a glycerol assembly procedure according to Shelanski et al. (1973) contains much less nontubulin proteins than tubulin isolated in the absence of glycerol by the procedure of Borisy et al. (1975). An interesting observation is the fact that a protein which shows the same electrophoretic mobility as EAT cell actin is greatly enriched in microtubule protein purified without glycerol. It is not yet clear if this result is significant; however, there is some evidence that actin may interact with tubulin under appropriate polymerization conditions (Griffith & Pollard, 1978). The effect of glycerol on the composition of microtubule protein and on the assembly reaction is still unclear. It has been suggested that this solvent can diminish the effects of possible assembly inhibitors (Nagle et al., 1977) or may lower the critical protein concentration by acting through a nonspecific solvent effect on the chemical potential of tubulin (Lee et al., 1978). We have found that it is very important for good yields in the polymerization steps to increase the tubulin concentration by preparing small and highly concentrated cytosol volumes. By this method, spontaneous microtubule assembly in EAT cell extracts does occur even in the absence of glycerol.

EAT cell tubulin purified by two cycling steps in the absence of glycerol contains, besides actin, several other nontubulin proteins. These components are not completely separable from tubulin by treatment with phosphocellulose. By means of chromatography on DEAE-Sephadex, microtubule protein could be separated into two fractions containing nontubulin proteins and into one fraction consisting of purified tubulin, as has been checked electrophoretically. The tubulin fraction readily produces microtubules in Pipes buffer without adding glycerol or increasing the magnesium concentration. This result indicates that the ability to form microtubules is probably an intrinsic property of tubulin itself. Our interpretation is in agreement with that of Lee & Timasheff (1975) who concluded that the basic information required for neurotubule formation seems to be present in the tubulin molecule. The fractionated nontubulin proteins, containing prominent polypeptides with molecular weights of about 70000, 49000 and 30000, did not significantly increase microtubule assembly when mixed with pure tubulin. From this result we conclude that these proteins are not required for tubule polymerization. Furthermore, EAT tubulin does not seem to contain the high molecular weight MAPs or τ factors found in neurotubulin. The identity and function of a distinct "nontubulin" protein, observed in reduced and carboxymethylated preparations of "pure" tubulin, are not yet known. However, we cannot exclude its possible role as a microtubule accessory protein and are currently testing this possibility by trying to isolate and to characterize this component.

The present study strongly indicates that DEAE-purified tubulin from EAT cells can readily assemble into microtubules in the absence of M_r(h) or τ-like MAPs and glycerol. Whether these results have any significance for the in vivo properties of microtubules is still difficult to answer. There is some evidence from immunofluorescence experiments that MAPs may be involved in the in vivo regulation of microtubules (Conolly et al., 1977, 1978; Sherline & Schiavone, 1978). Further work has to be done to support the possible significance of these observations and to identify which protein, if any, may regulate microtubule assembly in vivo.

Acknowledgments

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Binding of Immunoglobulin G to Phospholipid Vesicles by Sonication†

Leaf Huang* and Stephen J. Kennel

ABSTRACT: Purified goat immunoglobulin G (IgG) does not bind to sonicated phospholipid vesicles. However, when IgG is sonicated together with phospholipids, 4–40% of the IgG can be bound to the vesicles, depending on the experimental conditions. The extent of binding depends on the period and power of sonication, the IgG to lipid ratio, and the lipid composition. Anionic phospholipids such as phosphatidylglycerol and phosphatidylserine, but not cholesterol, enhance binding about 50% over that obtained with the neutral phosphatidylcholine. Binding of IgG causes extensive aggregation of vesicles, as shown by electron microscopy, and aggregates can be separated from unbound IgG by molecular-sieve chromatography on Sepharose 4B or by sucrose density gradient centrifugation. The IgG–vesicle aggregates remain stable in either phosphate-buffered saline or 50% fetal calf serum up to 20 h at 37 °C, although substantial lipid degradation in 50% fetal calf serum was observed. The use of goat IgG containing antibody to a purified protein antigen allowed quantitation of antibody activity of these preparations. Immune IgG sonicated alone shows 100% of the original antigen binding capacity while vesicle-bound IgG retains 30–50%. Antigen binding capacity of bound IgG is not increased when vesicles are lysed by 1.5% NP-40, suggesting all of the bound IgG is exposed on the outer surfaces of the vesicles. IgG's of human, mouse, and rabbit, as well as the purified goat $F(ab')_2$ fragments, also bind with vesicles by cosonication.

Phospholipid vesicles (liposomes) have been used extensively both to modify the plasma membrane composition and cell behavior and as carriers for drugs, hormones, enzymes, or other biologically active substances in vitro and in vivo (see reviews, Pagano & Weinstein, 1978; Gregoriadis, 1976a,b). One of the central problems of this area of research is the lack of specificity of the interaction between phospholipid vesicles and cells. It is particularly serious for in vivo experiments in which intravenously injected vesicles are rapidly cleared from circulation by the reticuloendothelial cells in liver and spleen (Juliano & Stamp, 1975; Gregoriadis & Neerunjun, 1974), thus preventing the effective use of vesicles as pharmaceutical carriers for other target tissues. Attempts have been made to modify the vesicle surface membranes with some "recognition" molecules so that vesicles can acquire specificity in terms of the type of tissue with which they interact (Gregoriadis & Neerunjun, 1975; Weinstein et al., 1978). Among all "recognition" molecules, immunoglobulins are preferred because of their ease of preparation, relatively high binding constants, specificity of recognition of target molecules, and ability to bind different antigen molecules. It has been shown that phospholipid vesicles coated with IgG[1] antibody can deliver their entrapped contents to target cells significantly better than uncoated vesicles (Gregoriadis & Neerunjun, 1975). Vesicles coated with heat aggregated IgM also cause

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[1] Abbreviations used: IgG, immunoglobulin G; PC, L-α-phosphatidylcholine; PG, α-phosphatidyl-DL-glycerol; chol, cholesterol; TLC, thin-layer chromatography; gp70, major envelope glycoprotein of Maloney leukemia virus; PBS, phosphate-buffered saline; αBGT, α-bungarotoxin; NP-40, Nonidet P40.

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