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THE ASSEMBLY OF TUBULIN INTO MEMBRANES

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ABSTRACT

Tubulin in high-speed supernatants of brain undergoes an alternate form of polymerization into structures that resemble membranes rather than microtubules. The reaction required elevated temperature (37°C) and was prevented by 1mM $CaCl_2$ or 10^{-4} M maytansine. The membraneous material was composed of tubulin (80%) and microtubule-associated proteins (8%) and contained phospholipids. The tubulin was identified on the basis of comigration in two-dimensional gel electrophoresis and colchicine-binding activity.

Tubulin, the subunit protein of microtubules, has been identified as a component of certain cell membranes on the basis of either colchicine-binding activity, electrophoretic analysis or peptide mapping evidence. The membranes reported to contain tubulin include synaptosomal membranes (1-4) cervical-ganglia plasma membranes (5), pigeon erythrocyte membranes (6), brain and thyroid membranes (7), and ciliary and flagellar membranes (8-11). The ciliary and flagellar membranes are unique in that as much as 60-80% of the total membrane protein is tubulin (8-11). The presence of tubulin as the major protein of certain membranes suggested that an alternate form of assembly for tubulin into membranes rather than microtubules could take place under the appropriate conditions. In this report, we show that under *in vitro* conditions a fraction of the soluble tubulin in brain extracts will assemble with lipid to form membranes.

MATERIALS AND METHODS

Tubulin was assembled from high-speed (100,000g x 1h) supernatants of bovine brain prepared by the methods of Borisy (11). The formation of membranes is inhibited by trace quantities of detergents so that all glassware used must be adequately rinsed. The assembly buffer contained 100 mM PIPES, pH 6.95, 1 mM EGTA, 0.1 mM $MgCl_2$ and 0.1 mM GTP. Colchicine at a concentration of 10^{-4}M was added to the cold extract prior to incubation in the dark at 37°C for 30 min. After incubation, the extract was kept in ice for at least 10 min. The cold extract was then centrifuged at 39,000 g x 30 min at 4°C. The pellet was homogenized in water and centrifuged through a continuous gradient of sucrose from 0.8M to 1.2 M, using an SW27 Beckman rotor at 25,000 RPM for 90 min at 4°C.

Polyacrylamide gel electrophoresis (either one- or two-dimensional) was performed using a modification of the methodology of O'Farrell (12)

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as reported previously (13). For electron micropscopy, the pellets were fixed in 2.5% glutaraldehyde, post-fixed in 1% O_s O_4. Colchicine-binding activity was determined by a DEAE filter paper assay for soluble proteins and by sedimentation through 10% sucrose for insoluble proteins (1).

RESULTS

When high-speed supernatants of bovine brain prepared in assembly buffer are warmed, tubulin polymerizes into microtubles (14). We assumed that tubulin might also assemble into other structures and prevented the assembly of microtubules with the combination of 10^{-4}M colchicine and cold depolymerization. Under these conditions, a gradual increase in the turbidity of the supernatant occurred during the incubation (Table I), indicating that larger aggregates had formed (15). Centrifugation at 20% of the total g x h used to prepare the initial supernatant resulted in the removal of most of these aggregates. Polyacrylamide gel electrophoresis of the sedimented material showed that it was composed predominantly of three proteins which co-migrated with α- and β-tubulin and actin (Figure 1b). Densitometry showed that α- and β- tubulin and actin accounted for 75% of the protein on the gel; these proteins were present in almost equal quantities. In addition, 8% of the protein corresponded in size and isoelectric mobility with the high-molecular weight microtubule associated proteins (14). In the electron microscope this material consisted predominantly of two components: membrane fragments and a granular material (Figure 1a). These components were separated by centrifugation into a continuous gradient of

TABLE I Absorbance during incubation

	100,000g x 1h	260,000 g x 1h
Initial absorbance	0.598	0.576
After 1 h at 37°C	0.646	0.632
After 39,000g x 30 min at 4°C	0.605	0.581

A high-speed supernatant of a brain homogenate was prepared by centrifugation at 100,000g x 1h; one-half of this supernatant was then centrifuged again at 260,000g x 1h. Both supernatants were incubated at 37°C in the presence of 10^{-4}M colchicine and the absorbance at 45nm was monitored. After incubation for one hour, during which the absorbance gradually increased as indicated, the supernatants were placed in ice for 15 min and then centrifuged at 39,000g x 30 min at 4°C. The absorbance of the supernatants was remeasured. The yield of assembled material was equal in both supernatants and identical in protein composition and morphology.

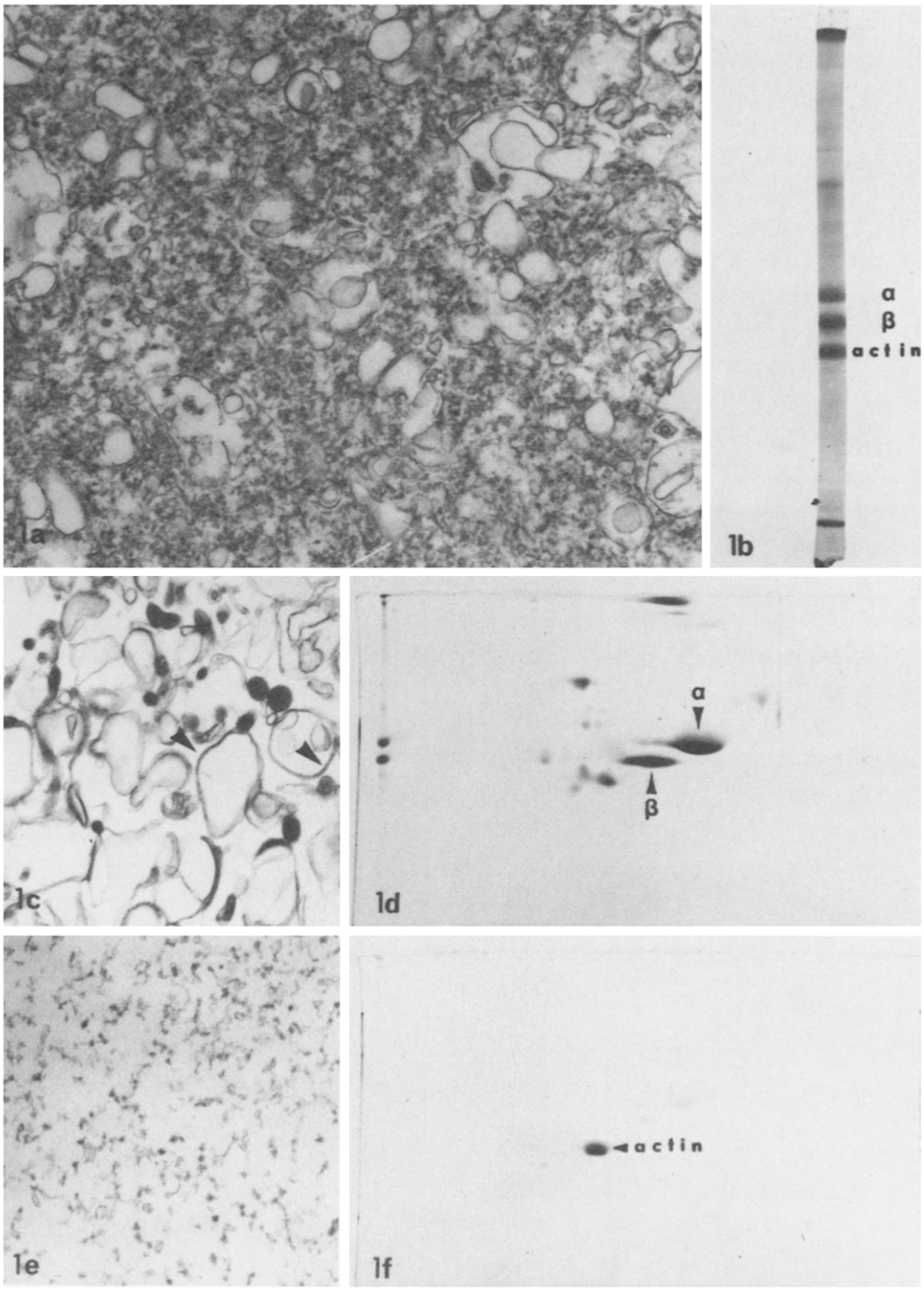

Figure 1:

(a) Electron micrograph (26,500×) of the membrane/granular fraction. (b) NadodSO$_4$-urea polyacrylamide gel electrophoresis of the membrane/granular fraction. In a separate experiment the bands co-migrated with α- and β-tubulin and actin as indicated.

sucrose from 0.8 M to 1.2 M. All of the protein was recovered in two closely spaced bands; the upper band consisted mainly of the granular material (Figure 1e) and the lower band contained membranes and dense spherical bodies (Figure 1c). The membranes and spherical bodies often appeared to connect with each other. In control experiments, tubulin precipitated with trichloroacetic acid or vinblastine was found to sediment through 1.2 M sucrose. The protein composition of the two fractions separated by density gradient centrifugation was determined by two-dimensional electrophoresis. The major protein (over 80%) in the fraction composed predominantly of membranes was tubulin with smaller amounts of actin and other proteins also present (Figure 1d). The granular fraction contained actin as the major protein and lesser amounts of tubulin (Figure 1f). No differences could be detected by two-dimensional electrophoresis between the tubulin in these fractions and tubulin prepared by 3 cycles of assembly into microtubules.

Two different physical criteria, centrifugation at lower force and increase in turbidity, indicated that during incubation in the presence of colchicine particulates were either forming or becoming larger. If the supernatant was centrifuged at higher force (260,000 g x h) the amount of material that assembled upon incubation was not decreased (Table I). There was no increase in optical density nor was a pellet obtained by centrifugation from control samples that were kept at 0° or if 1mM $CaCl_2$ was substituted for EGTA. Maytansine (10^{-4} M) alone or in the presence of colchicine prevented the formation of both membranes and the granular material. Thus the membranes and granular material which were formed during incubation derived from smaller subunits which did not sediment at 260,000g; whether these precursor subunits were protein molecules, protein aggregates or protein-lipid aggregates is still to be determined.

The conditions used in these experiments for the assembly of membranes that contain tubulin are probably not optimal. In particular,

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(c,e) Electron micrographs (40,000×) of the two fractions obtained by centrifuging the membrane/granular material through a continuous sucrose gradient that varied from 0.8M to 1.2M. The lower band on this gradient, containing the membraneous material, is shown in (c) and the upper band, containing the granular material, is shown in (e).

(d,f) Two dimensional polyacrylamide gel electrophoresis of the membraneous material (d) and granular material (f). The identity of the spots was determined by mixing experiments with the appropriate standards. The positions of α- and β-tubulin and actin are indicated. The first dimension (isoelectric focusing) is along the horizontal axis and the second dimension (NadodSO_4-urea polyacrylamide gel electrophoresis) is in the vertical direction.

TABLE II Phospholipid content

	μg phospholipid/mg protein
Membrane/granular fraction	31.9
Microtubules	< 1.5
Synaptic plasma membrane	928.0

Phospholipid content of tubulin-containing fractions. 15 mg of the membrane/granular fraction and of twice-polymerized microtubules were each extracted twice with 10ml of chloroform-methanol, 2:1 v/v, and the extract was washed three times with water. The phosphorous remaining in the organic phase was determined. A similar determination for the synaptic plasma membrane (24) is included.

the assembly of tubulin into membranes appears to require lipid. The assembled membranes have the classic trilaminar appearance of cell membranes (Figure 1c arrows) and are presumably protein-lipid bilayers. However, some purified preparations of tubulin have been reported to contain phospholipids (16,17). We therefore compared the lipid phosphorous in the membrane/granular fraction and in twice polymerized microtubules (Table II). The membrane/granular fraction contained more phospholipid than twice-reassembled microtubules but less phospholipid than the synaptic plasma membrane (Table II). How this lipid is distributed among the various components of the membrane/granular fraction is not known, and the lipid content of the membranes in this fraction may be considerably larger.

Since the membranes were assembled in the presence of colchicine, it was important to determine if tubulin inactive for colchicine-binding assembled into membranes. The membrane/granular material was polymerized from the brain extracts in the presence of 10^{-4}M colchicine, collected by sedimentation, and then incubated for 90 min at 37°C with [^{3}H] colchicine (at a concentration of 10^{-6}M). Parallel experiments were performed with microtubules. After the incubation, the material that remained assembled was collected by centrifugation. The protein that was released during incubation had a very high colchicine-binding activity whereas the assembled protein had a very low colchicine-binding activity (Table III), although the protein composition of both the released and assembled material was the same as is shown in Figure 1B. Similarly, at 10^{-6}M colchicine concentration microtubules that have been prepared by assembly are partially depolymerized (15); the depolymerized tubulin had a very

TABLE III Colchicine-binding activity

	cpm/mg protein
Depolymerized microtubules	506,000
Polymerized microtubules	22,000
Depolymerized membranes/granular fraction	422,000
Polymerized membranes/granular fraction	11,000

Microtubules were prepared by one round of assembly of the initial brain extract. The membrane/granular fraction was prepared by polymerization of the initial brain extract in the presence of 10^{-4}M colchicine, and depolymerization at 0°C. The fractions were collected by centrifugation (39,000g x 30 min) at 30°C and 4°C, respectively. The microtubules (3.3 mg/ml) and membrane/granular fractions (1.7 mg/ml) were suspended in assembly buffer and incubated for 90 min at 37°C with 1 μmole/ml of [^{3}H] colchicine (specific activity 16 mCi/μmole). Both samples were then centrifuged (39,000g x 30 min) at 30°C; the protein-bound radioactivity in the supernatant (the material depolymerized by colchicine) was determined by DEAE-filter paper assay (1) and the radioactivity in the pellets (the material that remained polymerized) was determined after sedimentation through 10% sucrose (1). The extent of depolymerization was 90% for the microtubules and 76% for the membrane/granular fraction. Polyacrylamide gel electrophoresis showed no difference between the protein released by colchicine and the protein that remained assembled. In the electron microscope, the material that remained assembled after treatment with colchicine was unchanged.

high colchicine binding activity whereas the assembled tubulin (composed almost entirely of microtubules) had much lower colchicine-binding activity (Table III). Therefore, intact microtubules and membranes that contain tubulin do not bind colchicine except perhaps at the edges or ends of these structures, but release colchicine-binding tubulin as they disassemble. These observations are consistent with other studies of colchicine-induced depolymerization of microtubules which have shown that the amount of depolymerization increased after fragmentation of the microtubules by brief sonication (15) and that in assembled microtubules most drug-binding sites are blocked (18).

DISCUSSION

We have observed that when brain extracts are warmed, a portion of the tubulin assembles into structures that are similar to membranes. This reaction is more readily apparent when the formation of microtubules is prevented by colchicine. The formation of these membrane-like structures requires elevated temperature and is accompanied by changes in two physical-chemical parameters (turbidity and sedimentation at 20%

of the initial centrifugal force used to prepare the supernatant before incubation) which indicate that an assembly reaction is occurring. The products of this reaction have unique sedimentation properties which distinguish them from vinblastine- or acid-induced aggregation of tubulin. The membraneous material also contains the characteristic high molecular weight microtubule associated proteins. These properties are consistent with the hypothesis that to a limited extent, an alternate form of assembly of tubulin into a membrane-like structure, rather than into a microtubule, takes place under the in vitro conditions employed. The extent of this reaction is probably severely limited by the small amounts of lipid present in the water-soluble extracts of brain. A specific interaction between purified tubulin and artificial liposomes has recently been reported (19). Further refinements in the conditions under which tubulin is polymerized in the presence of lipids should lead to an improved in-vitro system for the assembly of tubulin into a membraneous form.

The production of structures that appear to be membranes but are composed of almost entirely one protein is somewhat surprising. However, Stephens (8) has demonstrated that tubulin accounts for over 80% of the protein in ciliary plasma membranes. Further studies of the organization of the protein and lipid moieties in these in-vitro assembled membranes and comparison with other membranes that contain tubulin such as the ciliary plasma membrane and the synaptic plasma membrane should provide further insights into the interaction of the cytoskeleton and the cell membrane.

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