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Tubulin requires tau for growth onto microtubule initiating sites

(flagella/*in vitro* assembly/electron microscopy)

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ABSTRACT **Tubulin purified by phosphocellulose chromatography and free of accessory proteins will not form microtubules in the absence or presence of microtubule initiating sites (flagellar microtubules). The capacity for growth onto pre-existing "seeds" can be restored by the addition of small quantities of partially purified tau protein. Larger quantities restore the capacity for spontaneous assembly. These results suggest that tubulin requires tau for both initiation *and* growth of microtubules and that tau is incorporated into the microtubule throughout its length.**

The development of procedures for the assembly of microtubules *in vitro* has made possible the investigation of specific physiological factors required for microtubule polymerization (1–3). Recently, microtubule-associated protein factors have been described that stimulate tubulin assembly (4–7). One of these factors, designated tau, is associated with microtubules purified by repeated cycles of polymerization and depolymerization, and can be separated from tubulin by ion exchange chromatography on phosphocellulose (4). In the absence of tau, purified tubulin occurs as a 6S dimer and will not form microtubules; addition of tau restores the ability of the tubulin to polymerize. These results indicated that tau was required for *initiation* of assembly, but it was not clear whether tau was also required for subsequent *elongation* of microtubules.

In the present study, preparations of partially purified tau (8) were used to determine whether tau is necessary for microtubule elongation. This was tested by assaying the ability of tubulin to assemble in the presence and absence of tau onto the ends of flagellar microtubules, which act as seeds for the initiation of brain tubulin assembly (9–12). The results show that purified tubulin does not have the capacity to assemble onto the ends of flagellar microtubules. Addition of small amounts of tau restores the capacity for elongation, indicating that tau is necessary for growth as well as initiation. The kinetics of microtubule assembly as a function of tau concentration suggest that tau is required in stoichiometric rather than catalytic amounts for microtubule formation. We conclude that tau is incorporated into growing microtubules as an integral structural component and is therefore essential for both initiation of assembly and subsequent elongation.

MATERIALS AND METHODS

Preparation of Microtubule Protein, Tubulin, and Tau. Microtubule protein was purified from porcine brain by three cycles of polymerization and depolymerization by the method of Shelanski *et al.* (3) as modified by Weingarten *et al.* (13). Immediately prior to use, the purified microtubules were pelleted at 100,000 × *g* for 40 min and resuspended in purification buffer [0.1 M morpholinoethanesulfonic acid (Mes) buffer, pH 6.4, containing 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA), 1 mM GTP, 0.5 mM $MgCl_2$, and 1 mM mercaptoethanol]. A high-speed supernatant of depolymerized microtubules was prepared by cooling the protein at 5 mg/ml in the above buffer at 0° for 30 min and sedimenting the solution at 190,000 × *g* for 2 hr. Phosphocellulose-purified tubulin was prepared as described (4, 8). Microtubule protein in purification buffer was repolymerized and centrifuged at 100,000 × *g* for 40 min at 28°. The resulting pellets were resuspended in Mes-EDTA buffer (25 mM Mes, pH 6.4, 0.5 mM $MgCl_2$, 0.1 mM EDTA, and 1 mM mercaptoethanol) to a final concentration of 8–10 mg/ml. After 10 min at 0° the solution was clarified by centrifugation at 12,000 × *g* for 10 min and applied to a phosphocellulose column (Whatman, batch 511) that had been washed and equilibrated in the same buffer (4). The loading concentration was less than 4 mg of protein per ml of phosphocellulose and the flow rate was less than 2 column volumes per hr. The unadsorbed fraction was brought to 0.1 M Mes, 2 mM EGTA, and 1 mM GTP and is henceforth referred to as phosphocellulose-purified tubulin (PC-tubulin). It was prepared immediately before each experiment since it was stable as assayed by polymerization for only about 6 hr at 0° in purification buffer. The efficiency with which the tubulin accessory proteins are removed and the flow rates that are attained in the columns are reproducible within a given batch of phosphocellulose but vary with the source of the phosphocellulose and even differ from batch to batch from the same manufacturer. It is therefore necessary to determine for each batch the optimum loading concentration and the resulting efficiency of purification.

Tau protein was purified from the fraction adsorbed to phosphocellulose. Microtubule protein (375 mg) was loaded on a 45-ml phosphocellulose column and washed with 2 column volumes of Mes-EDTA buffer. The fraction containing tau was eluted with a single step of buffer containing 0.8 M NaCl. This fraction, containing 30 mg of protein, was desalted on a Bio-Rad P-6 column into purification buffer, and 13 mg was applied to a 4.5-ml hydroxyapatite column (Bio-Rad) equilibrated with purification buffer. Bound protein was eluted with a gradient of 0–0.45 M KH_2PO_4 in purification buffer. Approximately ¾ of the activity eluted in a peak centered at 0.09 M KH_2PO_4 containing 3 mg of protein. This protein was again desalted into purification buffer and stored frozen at −20°. The activity remains stable for up to 8 months. The proteins were monitored for activity during the purification as described elsewhere (ref. 8, and Cleveland, Hwo, and Kirschner, in preparation). Protein concentration was determined by the method of Lowry *et al.* (14) with bovine serum albumin as a standard.

Gel Electrophoresis. Sodium dodecyl sulfate gel electrophoresis was performed on slab gels by the method of Laemmli (15), except that an 8.5% running gel was used and the running buffer was adjusted to pH 9.1. For quantitative analysis, the gels were stained with fast green and traced with a Joyce-Loebl microdensitometer (16).

Preparation of Axonemes. *Chlamydomonas reinhardii*

Abbreviations: Mes, morpholinoethanesulfonic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; PC-tubulin, tubulin purified by chromatography on phosphocellulose.

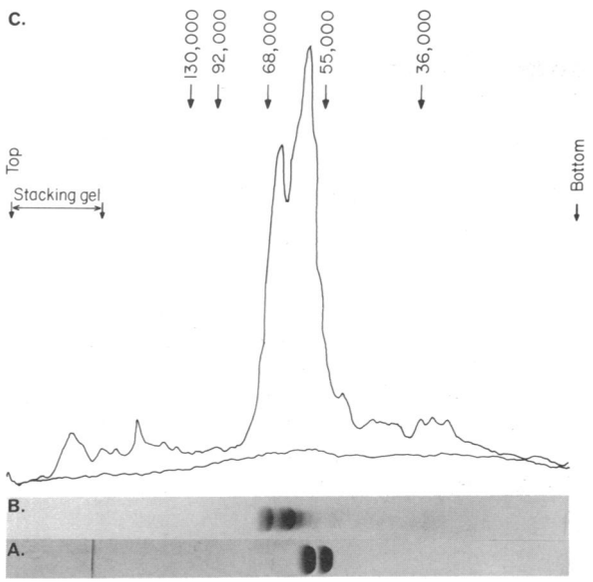

FIG. 1. (A) Sodium dodecyl sulfate gel of PC-tubulin stained with Coomassie blue. The line near the top of the gel is the interface between the running and stacking gels and does not represent protein. (B) Sodium dodecyl sulfate gel of tau protein stained with fast green. (C) Densitometer tracing of gel B. Gel boundaries are marked and the positions of migration of proteins of known molecular weight are shown. The bands migrating between 58,000 and 66,000 molecular weight constitute greater than 70% of the total protein. The lower tracing was made through a portion of the gel that contained no protein and was used as a baseline for quantitation.

(strain 1132D) was grown in synchronous culture as described (17). Cells in the sixth hour of the light period were harvested as described (18) and washed twice with 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) buffer at pH 7.8. Flagella were then detached and demembranated by treatment with 0.04% Nonidet P-40, and the axonemes were isolated, as described by Allen and Borisy (9).

Turbidometry. Turbidometric assays of microtubule polymerization were done at 380 nm with 1-cm path length curvettes at 37° in a thermostatically controlled cell compartment of a Cary 14 spectrophotometer. Polymerization of various tau-tubulin mixtures was initiated by the addition of GTP to a concentration of 1 mM.

Electron Microscopy. Samples were drop loaded onto carbon coated over Collodion 400 mesh copper grids. After 30 sec the drop was removed with filter paper and replaced in rapid succession with 2 drops of 2% uranyl acetate and drained dry. Quantitative electron microscopic analysis was performed by a modification (19) of the method of Backus and Williams (20). The sample to be analyzed was mixed with 0.1 mg/ml of tomato bushy stunt virus (a kind gift of Prof. C. A. Knight, Univ. of Calif., Berkeley, Calif.) and spray deposited by means of a specially designed double nebulizer (19) onto a glowed grid. This procedure produced droplets having a volume of about 10^{-11} ml. Each droplet was then photographed in its entirety at a magnification of 6000–8000, and the amount of microtubules was measured and expressed relative to the number of bushy stunt virus particles in the drop. Specimens were examined on a JEOL 100C electron microscope.

RESULTS

Purity of Tau and Tubulin Preparation. Fig. 1 shows a sodium dodecyl sulfate gel pattern from the purified tubulin (PC-tubulin) (Fig. 1A) and tau fraction (Fig. 1B) used in these experiments. The tubulin fraction contained α- and β-tubulin and no other observable peptides on overloaded gels stained with Coomassie blue. The tau fraction consisted primarily of two major peptides and two minor peptides having molecular weights between 58,000 and 66,000. Quantitative densitometry of gels stained with fast green indicated that these proteins represented over 70% of the total protein in the tau fraction (Fig. 1C). No other peptide amounted to more than 3% by weight.

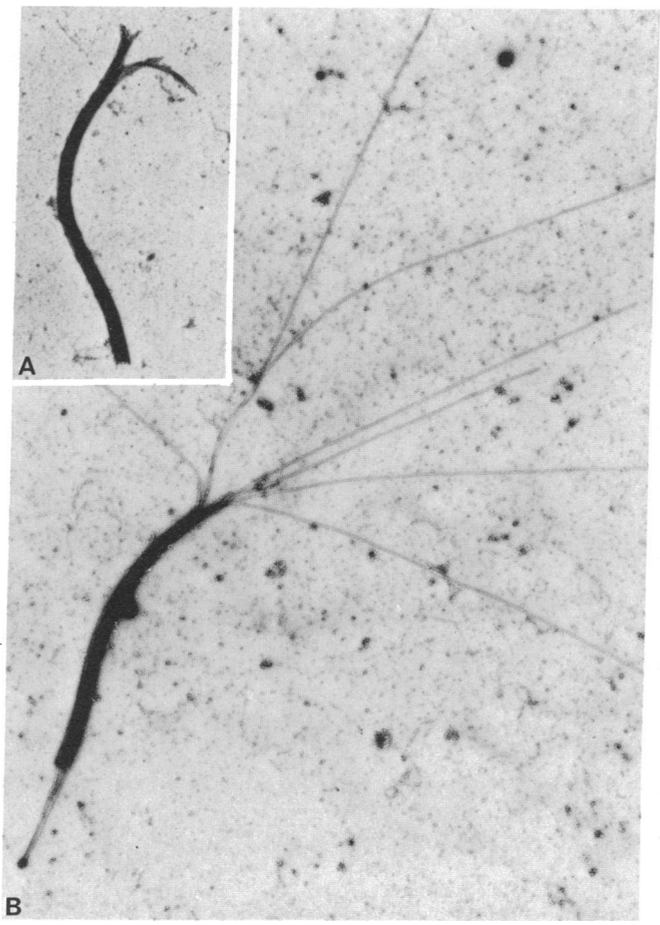

FIG. 2. (A) Negatively stained axoneme which was incubated for 20 min at 37° in reassembly buffer containing PC-tubulin (0.5 mg/ml) but no tau. No addition of microtubules was observed at either end. In such isolated, negatively stained axonemes, the flagellar microtubules separate from each other at the distal end (top of micrograph) but remain closely associated at the proximal end (9, 17). ×3720. (B) Axoneme incubated as in part A but with 0.03 mg/ml of tau protein added. Extensive elongation occurred at the distal tip of the axoneme; growth also occurred at the proximal end, but was not as great. ×3720.

Tau and Tubulin Addition onto Microtubule Initiating Sites. To determine if tau is necessary for microtubule elongation, we investigated the ability of tubulin from which tau had been removed by phosphocellulose chromatography (PC-tubulin) to assemble onto existing microtubules in the presence and absence of tau. Recent studies showed that 6S tubulin obtained by high-speed centrifugation of brain extracts or depolymerized microtubules will not initiate microtubule formation but, under the same conditions, will polymerize onto the ends of flagellar microtubules (9–12). These observations were confirmed in the present study. Tubulin (1.1 mg/ml) in high-speed supernatants assembled onto both the proximal and

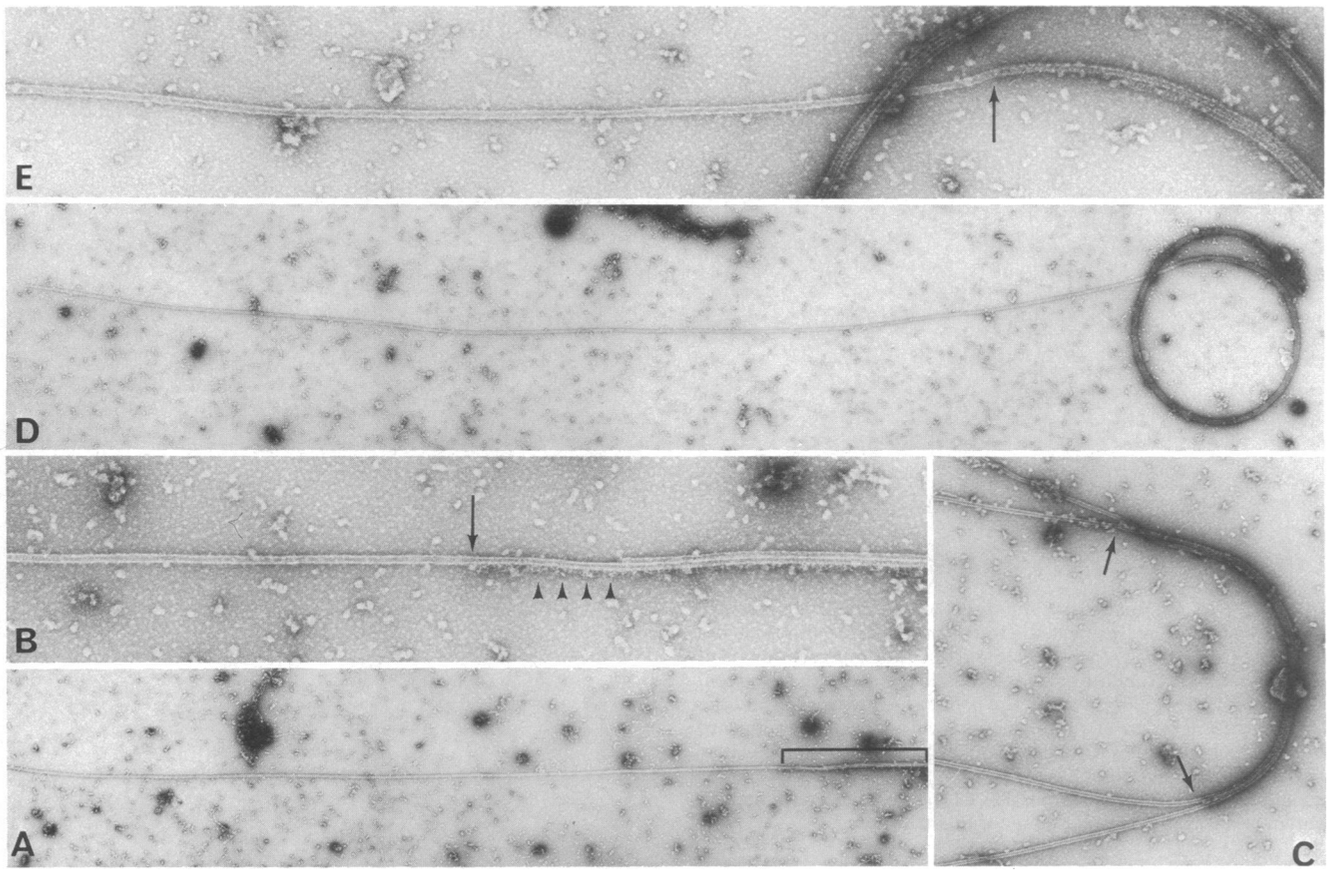

FIG. 3. Electron micrographs of flagellar microtubules incubated as described in legend of Fig. 2B. (A) Portion of an outer doublet (bracket) with more than 12 μm of added neurotubule. (B) Higher magnification of region in panel A showing continuity of the A-tubule of the outer doublet with the neurotubule at the point indicated by the arrow. The A-tubule is clearly recognized because it extends beyond the termination of the B-tubule, stains darker than the neurotubule, and has pairs of radial spokes (arrowheads) (9, 10). (C) Fragment of a central tubule pair with neurotubule growth at both ends (arrows) of both tubules. The central tubules are easily distinguished from the neurotubules by their darker staining and the presence of striations, which represent elements of the central sheath (9, 10). (D) Central tubule pair with more than 17 μm of added neurotubule. (E) Higher magnification of portion of panel D showing the transition (arrow) from the more darkly staining central tubule to the more lightly staining neurotubule. (A and D) ×8960; (B and E) ×30,700; (C) ×15,350.

distal ends of central and outer doublet microtubules of isolated *Chlamydomonas* axonemes. The neurotubules were not formed in the absence of GTP or in the presence of 5 mM colchicine, and were depolymerized by treatment at 0° for 15 min or with 2 mM $CaCl_2$.

In contrast to these results obtained with 6S tubulin prepared from high-speed supernatants of depolymerized microtubules, PC-tubulin at concentrations ranging from 0.2 to 3 mg/ml did not add onto flagellar microtubules (Fig. 2A). However, when low concentrations of tau were added to suspensions of PC-tubulin and flagellar axonemes, extensive growth was observed on both the proximal and distal ends of the axonemes (Fig. 2B). The added neurotubules were morphologically indistinguishable from normal neurotubules. They formed only on addition of GTP and were depolymerized by addition of 2 mM $CaCl_2$ or cooling to 0°. At higher magnifications, it was clear that the neurotubules were continuous with the flagellar microtubules. Growth was observed only from the A-tubules (Fig. 3A and B) and from both ends of fragments of both central pair tubules (Fig. 3 C–E). When axonemes were incubated with tau alone, no growth was observed.

At low concentrations of tau protein preparations (0.03 mg/ml) and with low concentrations of PC-tubulin (0.5 mg/ml), extensive growth was still observed on the flagellar microtubules, but very few neurotubules were seen that were not attached to ends of flagellar axonemes. The minimum tau concentration in which neurotubular growth was observed was 0.01 mg/ml. At higher concentrations of tau (0.16 mg/ml) or higher concentrations of tubulin (1–3 mg/ml), large numbers of background neurotubules were observed in addition to those which grew onto the flagellar microtubules.

Effects of Tau on Rate and Amount of Tubulin Polymerization. The effect of various amounts of tau on tubulin assembly was studied by turbidometry, which is thought to measure total polymer mass for objects the shape and size of microtubules (21, 22). Solutions of PC-tubulin showed no increase in turbidity upon addition of GTP and incubation at 37°. When increasing amounts of tau were added to PC-tubulin, there was an increase in both the initial rate of the turbidity change and in the final amount of turbidity when it had reached a plateau value (Fig. 4A). As shown in the insert to Fig. 4A, the plateau values of turbidity increased with increasing tau concentration until saturation was achieved. These results indicated that both the rate of assembly and the yield of microtubules varied directly with the concentration of tau.

Similar results were obtained when microtubule assembly was monitored directly by quantitative electron microscopy, which has the advantage that microtubules can be distinguished from any other type of tubulin aggregate (see *Materials and Methods*). By this assay, an increase in both the initial rate of assembly and the total mass of microtubules formed was again found with increasing amounts of tau (Fig. 4B). No microtub-

ules or other polymeric forms were observed when PC-tubulin was incubated in the absence of tau.

DISCUSSION

Electrophoretic analysis of tubulin purified by phosphocellulose chromatography showed that it contained only the α- and β-subunits of tubulin; no other bands were observed even in highly overloaded gels. Electrophoretic analysis of the tau fraction obtained by hydroxyapatite chromatography showed that it contained two major bands and two minor bands having molecular weights between 58,000 and 66,000. These proteins comprised over 70% of the total protein in this fraction and were the only proteins present in significant amounts. All four tau proteins have very similar peptide maps and amino acid compositions and may be modified versions of the same polypeptide chain (Cleveland and Kirschner, unpublished results). High molecular weight peptides ($\geq$200,000) are not found in this fraction but elute from hydroxyapatite at higher salt concentrations (about 0.3 M).

PC-tubulin in the absence of tau exists entirely as a 6S dimer and does not form rings or other intermediates of tubulin assembly, indicating that tau is necessary for the initiation of microtubule polymerization (4). In the present study, tau was shown also to be required for microtubule elongation by experiments involving incubation of PC-tubulin with microtubule initiating sites. Purified tubulin alone did not add onto flagellar microtubules, even though such microtubules were capable of serving as "seeds" or nucleation centers for the assembly of 6S tubulin prepared by high-speed centrifugation of depolymerized brain microtubules. However, addition of tau completely restored the capacity of PC-tubulin to assemble onto flagellar microtubules. These experiments clearly demonstrated that 6S tubulin from which tau has been completely removed by ion exchange chromatography is insufficient to support microtubule growth even in the presence of assembly initiating sites. From these results we conclude that tau is essential for microtubule elongation.

Tau might be necessary for microtubule elongation because it is an essential structural component of microtubules. In this case both the rate of assembly and the final yield of microtubules would be proportional to the amount of tau added. Alternatively, tau might in some way aid microtubule assembly without actually being incorporated into the final structure; in this case increased amounts of tau would increase the rate of assembly but not the final yield of microtubules. The results obtained in this study clearly showed that the yield of microtubules as well as the rate of assembly increased directly with the amount of tau protein present, as judged by turbidometric and quantitative electron microscopic assays. These findings suggest that tau is incorporated into the microtubule in direct proportion to tubulin. There may be a precise stoichiometry between tau and tubulin; alternatively, there may be a minimum requirement for tau but additional amounts could be incorporated into the microtubule lattice.

In several earlier studies it was shown that tubulin obtained by high-speed centrifugation or gel permeation chromatography did not polymerize or polymerized only slowly in the absence of pre-existing microtubule initiating sites, but rapidly assembled onto added seeds (5, 6, 9–12, 23). These results suggested that tubulin alone might be adequate for growth after initiation of assembly. However, since tubulin purified by phosphocellulose chromatography does not polymerize in the absence or presence of nucleating sites, it seems probable that the tubulin used in the earlier studies on microtubule elongation contained small amounts of accessory proteins such as tau. In

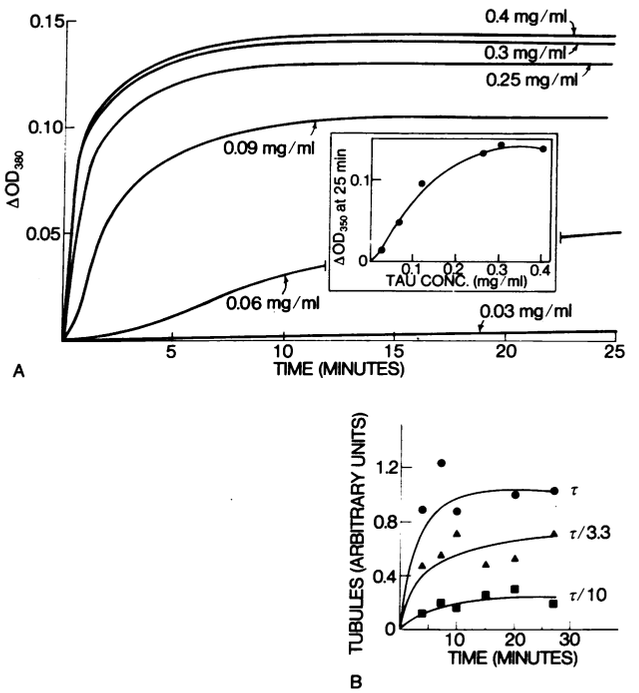

FIG. 4. Measurement of tubulin assembly. (A) Turbidometric assay. PC-tubulin (0.4 ml) at 1.8 mg/ml in reassembly buffer was diluted with an equal volume of tau at concentrations ranging from 0.06 to 0.8 mg/ml in the same buffer and incubated at 37° in a 1-cm light path quartz cuvette in a Cary 14 spectrophotometer. After 2 min, 10 μl of 0.1 M GTP was added and the optical density change at 380 nm recorded. Insert shows the 25-min plateau values for optical density change plotted versus tau concentration. (B) Quantitative electron microscopic assay. An aliquot of 0.25 ml of 4 mg/ml of PC-tubulin in purification buffer and 0.1 ml of a preparation of bushy stunt virus (1 mg/ml) were mixed with 0.5 ml of a preparation of tau protein (1 mg/ml) or with the same volume of a 1/3.3 or a 1/10 dilution of the tau preparation. Polymerization was then initiated by addition of 5 mM EGTA and 1 mM GTP and warming to 37°. Aliquots were removed at various times and drop patterns prepared as described in *Materials and Methods*. The results are expressed as a ratio of total microtubule length per unit volume in arbitrary units.

one of these studies, Murphy and Borisy (6) showed that the tubulin used in their experiments was greater than 95% pure. However, our results indicate that the presence of as little as 0.5% tau will permit microtubule elongation onto pre-existing microtubules, and that somewhat higher amounts of tau will also permit slow assembly in the absence of added nucleating sites (Fig. 4). Such low levels of tau probably would not have been detected by Murphy and Borisy in their electrophoretic analysis of the tubulin purified by their procedure.

Studies in several laboratories have shown that other proteins, which copurify with tubulin through cycles of polymerization-depolymerization, will stimulate microtubule assembly (5–7, 11). These proteins are found in varying amounts in different microtubule preparations (24). They differ from tau in their molecular weights, which have been estimated to be 270,000 or more. Furthermore, in contrast to tau, there does not appear to be an absolute requirement for these high-molecular-weight proteins in microtubule assembly. Tubulin lacking high-molecular-weight protein will assemble onto pre-existing microtubule nucleating sites or more slowly polymerize in the absence of added nucleating sites (5, 6, 25).

The ability of tubulin to aggregate has also been reported to be stimulated by the addition of polycations such as histones

and DEAE-dextran (26, 27), suggesting that positively charged macromolecules can interact with the negatively charged tubulin to permit aggregation in the absence of tau. However, the aggregates formed in this way have abnormal morphology and have been termed "duplex microtubules" (27), while the structures formed by the addition of tau are structurally normal microtubules. In addition, tau is a slightly acidic protein (Cleveland and Kirschner, unpublished observations) and therefore may promote assembly by a different mechanism.

Recently, Lee and Timasheff (28) have reported that highly purified tubulin can be induced to form microtubules at high tubulin concentrations and high levels of Mg^{++} (0.016 M) and glycerol (3.4 M). While these conditions are clearly nonphysiological, this finding suggests that the basic bonding geometry for forming the microtubule is built into the tubulin subunit itself. Under physiological conditions, tau is probably required to stabilize the intersubunit tubulin bonds.

In a previous report it was shown that tau remains associated with tubulin through repeated cycles of polymerization and depolymerization (4). In the present study it was determined that tau is required for microtubule elongation and that it acts in a stoichiometric rather than a catalytic manner. Together, these findings suggest that tau is incorporated throughout the entire length of the microtubule as an integral component of the microtubule structure.

Electron microscopic studies have shown that, during microtubule assembly, the 36S rings open and uncoil to form microtubule protofilaments (19, 29, 30). Hydrodynamic measurements indicate that tau is a long asymmetric molecule (8). Since tau is contained in and is necessary for the formation of 36S rings (4), it seems probable that tau stabilizes longitudinal bonding domains in the rings and in the protofilaments within microtubules. In the microtubule lattice, tau might then be located in a groove between adjacent protofilaments, where, in addition to stabilizing longitudinal interactions along the protofilament, it may also stabilize lateral bonding of the tubulin subunits between protofilaments.

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