Proc. Natl. Acad. Sci. USA
Vol. 76, No. 1, pp. 0000–0000, January 1979
Cell Biology

Self-assembly of microtubules in extracts of cultured HeLa cells and the identification of HeLa microtubule-associated proteins

(polymerization/tubulin)

JEANNETTE CHLOË BULINSKI AND GARY G. BORISY

Laboratory of Molecular Biology, University of Wisconsin, Madison, Wisconsin 53706

Communicated by David Marshall Prescott, September 29, 1978

ABSTRACT **Microtubule protein from HeLa cell extracts was purified by multiple cycles of polymerization and depolymerization in the absence of glycerol or other exogenous polymerization-stimulatory agents. Approximately 4–5% of the extract protein was tubulin, of which more than one-half was competent to participate in polymerization–depolymerization cycles. The purified HeLa microtubule protein preparations contained 95% tubulin after the second cycle of polymerization and depolymerization. Additional protein species bound specifically to and copurified quantitatively with microtubules throughout at least four cycles of polymerization and depolymerization. These microtubule-associated proteins (MAPs) were separated from tubulin by DEAE column chromatography. When added to purified brain or HeLa tubulin, these MAPs stimulated the polymerization of microtubules as assayed by electron microscopy and a quantitative sedimentation assay. The most prominent HeLa MAPs had molecular weights of approximately 210,000 and 120,000.**

An understanding of the regulation of microtubule formation in cells would be greatly facilitated by an *in vitro* system in which cultured cell tubulin could spontaneously assemble and in which potential regulatory molecules could be identified. The successful development of *in vitro* assembly procedures for brain tissue microtubules (1–4) has led to attempts to extend these techniques to the study of homogeneous cell populations in which there is greater potential for studying the mechanisms regulating assembly. The discovery of proteins specifically associated with the microtubules of brain tissue (5–7) and the immunofluorescent localization of crossreacting material in cultured cells (8–11) has led to the expectation that microtubule-associated proteins (MAPs) (12) are indeed of general occurrence. However, the development of a microtubule assembly system for cultured cells has not been straightforward, and as yet no cultured cell MAPs have been clearly identified.

A satisfactory assembly system should display the following properties: (*i*) Microtubule formation should occur spontaneously. It should not require the addition of heterologous factors to stimulate polymerization or the addition of microtubule fragments to serve as seeds. (*ii*) Microtubule formation should be truly reversible as evidenced by multiple cycles of assembly–disassembly. As indicated in our original method (4), a single cycle of assembly is insufficient proof of microtubule formation because of the possible irreversible formation of nonmicrotubular aggregates of tubulin. Additionally, one or two cycles may be insufficient to demonstrate the existence of a quantitatively copurifying MAP, because contaminants and weakly associated proteins may be incompletely removed. Demonstration of a quantitatively copurifying MAP requires establishment of a plateau in the ratio of MAP to tubulin in polymeric form upon successive cycles of assembly (13, 14). (*iii*) Self-assembly of cell tubulin should occur under conditions that favor the specific association of putative MAPs. Polymerization media containing high concentrations of glycerol might not be suitable for this kind of study because it has been shown that glycerol binds to tubulin (15) and reduces the yield of at least some MAPs (16, 17).

Nagle *et al.* (18) demonstrated polymerization in extracts from various cell lines. Their extracts contained inhibitors of assembly which could be overcome by the inclusion of 1–6 M glycerol in the assembly medium. Yields in their system were low and most samples were carried through only one cycle of assembly–disassembly. A MAP of molecular weight 49,000 was provisionally identified but conclusive identification was hampered by the inability to cycle the protein more than twice. Wiche *et al.* (19, 20) have reported polymerization of tubulin in a rat glial clone, C_6, and from mouse 3T3 cells but they too used glycerol and were hampered by poor yields when they attempted repeated cycles. While this manuscript was in preparation, a report of self-assembly of HeLa tubulin appeared (17) in which two cycles of polymerization–depolymerization were achieved and a putative MAP of molecular weight 68,000 was identified. However, the 68,000-molecular weight species was not demonstrated to copurify quantitatively.

Here we report conditions for the self-assembly of microtubules in extracts of HeLa cells. The polymerization meets the three criteria outlined above. It is spontaneous and reversible in good yield through four cycles of assembly–disassembly, and quantitatively copurifying proteins have been identified. These HeLa MAPs also stimulate the polymerization of pure HeLa or brain tubulin and cosediment with polymerized tubulin in a reconstituted system.

MATERIALS AND METHODS

Cell Culture and Preparation of Cell Extracts. HeLa cells, strain H, were obtained from the laboratory of R. R. Rueckert at the University of Wisconsin and grown as described by Medappa *et al.* (21).

Cells were harvested by centrifugation at 6800 × *g* for 5 min. Cells were washed three times with phosphate-buffered saline [lacking Ca^{2+} or Mg^{2+} (22)], once with 0.1 M Pipes [piperazine-*N*,*N*′-bis (2-ethane sulfonic acid)] (pH 6.94), and once with 0.1 M Pipes (pH 6.94), containing 1 mM dithiothreitol, 1 mM EGTA, 1 mM $MgSO_4$ and 1 mM GTP (PDEMG buffer). Cell pellets (typically 16-ml packed cell volume from 10 liters of culture) were combined with an equal volume of PDEMG and sonicated at setting 2 with a Heat Systems sonifier (Heat Systems–Ultrasonics, Plainview, NY). We centrifuged the lysate thus obtained at 48,000 × *g* for 30 min to produce a cell extract. All operations to this point were at 0–4°C.

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Abbreviation: MAP, microtubule-associated protein.

Preparation of Microtubule Protein, Tubulin, and MAP Fractions. HeLa and brain microtubule protein were prepared from extracts by the method of Borisy *et al.* (4), except that PDEMG buffer was used. To separate HeLa tubulin from MAPs, NaCl was added to thrice-cycled HeLa microtubule protein to a final concentration of 0.1 M NaCl in PDEMG buffer. This material was loaded onto a DEAE-Sephadex A-50 column preequilibrated with 0.1 M NaCl in PDEMG. The MAP fraction was the material that did not bind to the column, and HeLa tubulin was eluted from the column in 0.5 M NaCl in PDEMG. DEAE-purified brain tubulin was prepared by the method of Vallee and Borisy (23). The MAP fractions and purified tubulin were dialyzed against PDEMG before use.

Assay Methods. Protein concentrations were determined by the method of Lowry *et al.* (24), using bovine serum albumin as a standard. Polyacrylamide gels were prepared according to the method of Laemmli (25) and were stained with Coomassie blue by the procedure of Fairbanks *et al.* (26).

Colchicine-binding assays of HeLa cell and brain tissue extracts were performed according to the method of Borisy (27) using thrice-cycled brain microtubule protein and freshly prepared DEAE-purified brain tubulin as standards.

To quantitate polymerization in various samples, we used a modification of the sedimentation assay of Johnson and Borisy (28). Samples of 0.2 ml were polymerized for 30 min at 37°C and were centrifuged at 48,000 × *g* for 30 min at 37°C. Protein determinations and gel electrophoresis were performed on supernatants and resuspended pellets.

Specific viscosity changes of mixtures of cell extracts and polymerizing microtubule protein solutions were measured by using the method of Olmsted and Borisy (29).

RESULTS

In order to obtain self-polymerization of tubulin in extracts of suspension-grown HeLa cells, certain important modifications of the brain microtubule polymerization system of Borisy *et al.* (4) were necessary. We found that the success or failure of the various HeLa polymerization procedures was partly determined by our method of cell lysis. The two major problems encountered were (*i*) the presence of endogenous inhibitory activity for microtubule assembly and (*ii*) the low concentration of tubulin in the extract. We assayed inhibitory activity by mixing various concentrations of extract with a constant amount of purified brain microtubule protein and measuring polymerization by viscometry (29). Tubulin concentration was assayed by the binding of [^{3}H]colchicine in a filter assay (27) which was corrected for decay of binding sites (30). Extracts prepared by lysis in a glass-on-glass homogenizer contained no inhibitory activity such as that described by Bryan *et al.* (31), but they contained small (<12 mg/ml) total protein concentrations. Extracts prepared by lysing cells in a motor-driven, teflon-on-metal, low clearance homogenizer possessed greater protein concentrations (15–25 mg/ml) but did contain inhibitory activity. When compared to these homogenization methods, gentle sonication of cells produced less heating and foaming of the lysates and produced extracts with protein concentrations of 20–40 mg/ml. These extracts contained no inhibitory activity and were capable of spontaneous self-assembly of microtubules.

The apparently extreme dependence of HeLa extract self-assembly on protein concentration can be understood from the following considerations. From colchicine-binding determinations we calculate that 4.3% of the soluble extract protein is tubulin. As will be shown below, the equilibrium monomer concentration of HeLa microtubule protein under our conditions is 0.5 mg/ml. The concentration of extract protein below which there is no self-assembly is therefore 11.6 mg/ml. Assuming some loss of activity during preparation, the total protein concentration in the extract required for significant polymerization would be in excess of 12 mg/ml.

The importance of solution conditions to the yield of microtubules was also examined. The HeLa extract tubulin assembled poorly when EGTA was omitted from the polymerization buffer. No assembly occurred in the absence of dithiothreitol, although other aggregates containing tubulin were formed. Addition of dithiothreitol and EGTA were the only necessary modifications of the brain microtubule protein purification method (4) we used to prepare microtubule protein from HeLa extracts.

Polymerization products of HeLa cell extracts were monitored by electron microscopy, gel electrophoresis, and sedimentation analysis. Fig. 1 shows an electron micrograph of HeLa microtubules in the second polymerization cycle. The microtubules shown are indistinguishable from brain microtubules.

We used quantitative sedimentation (28) to determine the yield of polymer and extent of reversibility in each polymerization or depolymerization cycle. Table 1 shows data obtained for four cycles of HeLa cell microtubule protein polymerization and depolymerization. Values of HeLa tubulin polymerizability (70–80%) and reversibility (60–90%) were similar to values for brain tubulin undergoing multiple cycles with the same procedure (4). Table 1 also documents the high purity of tubulin achieved after two cycles of polymerization and depolymerization. In the last four purified samples, tubulin represented a constant 95% of the protein present, as quantitated on polyacrylamide gels.

The tubulin purified from HeLa cells was identical to brain tubulin in its electrophoretic behavior. They both possessed the same relative mobility on sodium dodecyl sulfate/polyacrylamide gels, and the HeLa α and β tubulin subunits could be resolved into two bands which comigrated with brain α and β tubulin.

We also measured the equilibrium monomer concentration of HeLa microtubule protein in our system by sedimenting the polymer and determining the concentration of tubulin in the supernatant. Colchicine-binding assays and protein determinations of the extract and all subsequent stages of purification gave a value of 0.5 mg of microtubule protein per ml.

We utilized gel electrophoresis to examine the composition of microtubule protein products at each stage of the purification

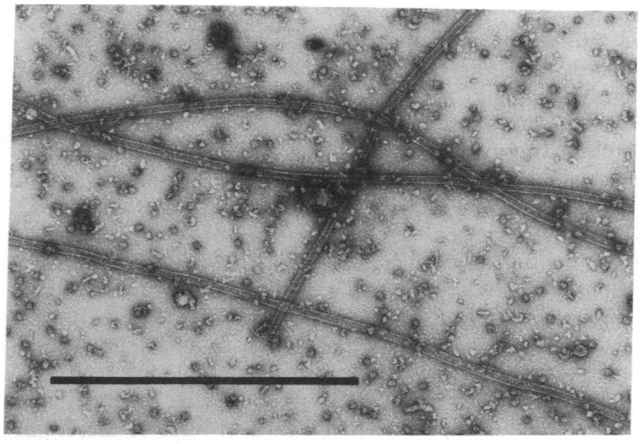

FIG. 1. Electron micrograph of HeLa microtubules. HeLa microtubule protein (cycled once) was polymerized for 30 min at 37°C; a 5-μl sample was applied to a Formvar–carbon coated grid, washed with three drops each of cytochrome *c* at 1 mg/ml and water, and negatively stained with 1% uranyl acetate. Bar indicates 1 μm.

Table 1. Reversible polymerization of HeLa cell microtubule protein

Sample*	% tubulin content†	% polymerization‡	% reversibility§
Extract	6.5	—	—
H_1P	25	26	—
C_1S	25	—	60
H_2P	40	70	—
C_2S	70	—	87
H_3P	95	83	—
C_3S	95	—	71
H_4P	95	85	—
C_4S	95	—	82

*Terminology in this table and elsewhere in this paper is that of Borisy *et al.* (4), in which H_XP denotes the pellet obtained from a 37°C polymerization and sedimentation. X is the cycle number. C_XS denotes the supernatant produced when an H_XP sample is depolymerized and sedimented at 0°C.

†The values for tubulin content shown in this column were determined by gel electrophoresis according to Laemmli (25) in order to separate α and β tubulins. When gel systems that did not separate α and β tubulins were used, gel electrophoresis overestimated tubulin content.

‡The extent of polymerization per cycle was determined as the ratio: 100 × (amount of tubulin in H_XP) to (amount of tubulin in $C_{X-1}S$).

§The extent of reversibility per cycle was determined as the ratio: 100 × (amount of tubulin in C_XS) to (amount of tubulin in H_XP).

procedure. Fig. 2 shows gel electrophoretic patterns of a HeLa extract and the microtubule protein products obtained during four cycles of temperature-dependent polymerization and depolymerization. In addition to documenting the purification of HeLa cell tubulin, these gels contain information about the nontubulin proteins that consistently copurified with HeLa tubulin. The molecular weights of these HeLa MAPs were established by reference to a standard curve prepared with monomer proteins of similar molecular weight including MAPs isolated from porcine brain tissue (Fig. 3).

Approximately 5% of the four-times-purified HeLa microtubule protein was present in two groups of species. One group contained a triplet of bands of molecular weights 220,000, 210,000, and 200,000. The second group contained a doublet of molecular weights 125,000 and 120,000. These proteins were

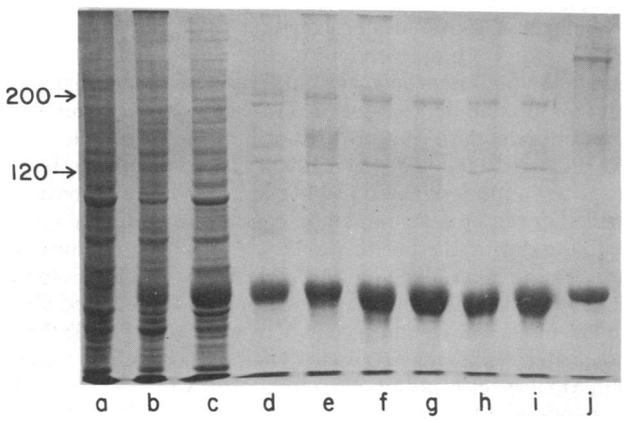

FIG. 2. Electrophoretic patterns of various stages of purification of HeLa microtubule protein. Lanes: a, HeLa extract (160 μg); b, H_1P (75 μg); c, C_1S (120 μg); d, H_2P (75 μg); e, C_2S (90 μg); f, H_3P (100 μg); g, C_3S (108 μg); h, H_4P (90 μg); i, C_4S (110 μg); j, brain H_3P (8 μg). Terminology is that of Borisy *et al.* (4). Gels (6.25%) were run by the method of Laemmli (25). The numbers 200 and 120 indicate the mobilities of species with molecular weights 200,000 and 120,000, respectively.

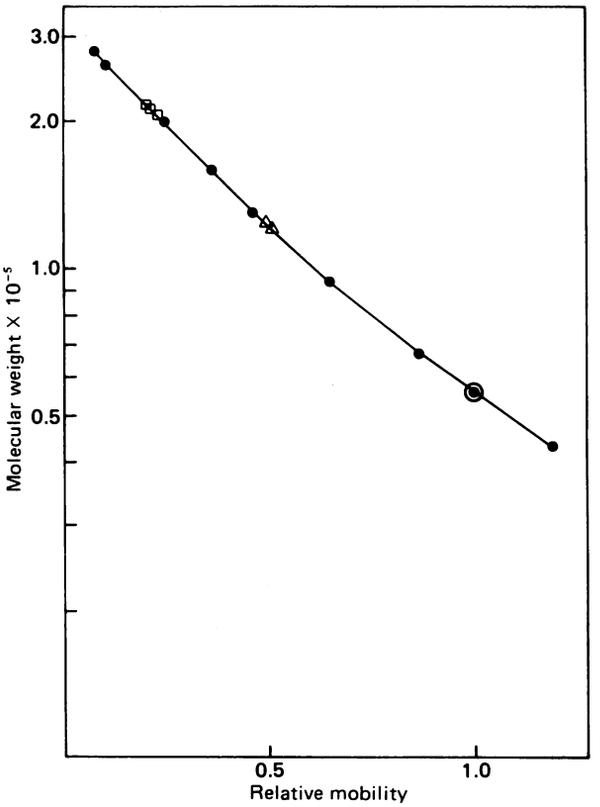

FIG. 3. Molecular weight determination of HeLa microtubule protein components on 7.5% Laemmli gels. ●, Mobility relative to porcine brain tubulin of various standards, which include actin, tubulin, bovine serum albumin, phosphorylase A, β galactosidase, RNA polymerase subunit β, myosin, and high molecular weight components of brain microtubule protein; ○, mobility relative to porcine brain tubulin of HeLa tubulin; △, relative mobility of the two lower molecular weight HeLa MAPs; □, relative mobility of the higher molecular weight HeLa MAPs.

barely detectable in the HeLa cell extract, were enriched in the first cycle of polymerization, and were present in constant amount relative to tubulin in all purified fractions after the first polymerization step. The gel pattern of MAPs in HeLa microtubule protein differed significantly from the gel pattern of brain MAPs. Only a trace component was present with a molecular weight above 220,000 in four-times-cycled HeLa protein, whereas the prominent brain MAP species, called HMW, have molecular weights of 286,000 and 271,000. A species of molecular weight 280,000 was detected in the HeLa extract but is not considered a MAP because it failed to copurify. In addition, we scrutinized the gel patterns for species of the molecular weight range(55,000–70,000) reported for tau (32, 33) and tubulin assembly protein (TAP) (11), other factors thought to be associated with microtubules. Components in this range were present in the extract and were relatively abundant. They were also present in once- and twice-cycled material but in diminished amounts and were not detectable in four-times-cycled protein.

We considered the possibility that the MAPs recovered in our purified HeLa microtubule protein preparations had been degraded to a lower molecular weight by proteases in the HeLa extract. This explanation was rendered unlikely for two reasons. First, addition of 1 mM concentrations of the protease inhibitors, *p*-tosyl-L-arginine methyl ester or phenylmethylsulfonylfluoride to our lysis and polymerization buffers did not alter the pattern of MAPS obtained through the fourth cycle of polymerization. Secondly, copolymerization experiments,

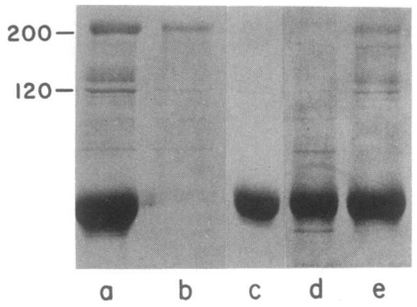

FIG. 4. Electrophoretic profiles of reconstituted HeLa tubulin and MAP mixture. Lanes: a, Unfractionated thrice-cycled HeLa microtubule protein, H_3P (150 μg); b, HeLa DEAE-purified MAP fraction (15 μg); c, HeLa DEAE-purified tubulin fraction (150 μg); d, supernatant obtained when HeLa DEAE-purified MAPs and DEAE-purified tubulin were reconstituted and polymerized and the microtubules were sedimented (80 μg); e, pellet obtained in the above sedimentation assay (100 μg). The numbers 120 and 200 indicate the mobilities of species with molecular weights 120,000 and 200,000, respectively. This gel was dried before photographing, and the semicircle across lanes d and e is an artifact of drying.

in which mixtures of brain microtubule protein and HeLa extracts were subjected to up to seven cycles of polymerization and depolymerization, showed the normal complement of brain MAPs, indicating the absence of protease activity for the known brain MAPs. This second experiment is thought to be particularly sensitive because the brain MAPs are known to be very susceptible to proteolysis, being degraded by as little as 0.16 μg of trypsin per ml within 15 min at 30°C (34).

In order to study the role of HeLa MAPs in the *in vitro* self-assembly of microtubules, we first separated HeLa microtubule protein into two fractions, a MAP fraction and a tubulin fraction. At a salt concentration of 0.1 M, the MAP fraction did not bind to DEAE-Sephadex A-50, whereas the tubulin bound almost quantitatively. After the DEAE column was washed free of MAPs, the tubulin was recovered by elution with 0.5 M salt. Fig. 4 shows that the MAP fraction thus obtained contained less than 10% tubulin. The tubulin was >99% pure; only trace impurities were visualized on gels loaded with up to 150 μg of protein.

The DEAE-purified MAP fraction was tested for its ability to stimulate microtubule polymerization. HeLa MAP fractions were mixed with HeLa or brain DEAE-purified tubulin under conditions in which self-assembly of the pure tubulin was negligible. The mixtures were incubated under polymerizing conditions and the polymerization products were monitored by electron microscopy and quantitative sedimentation assays.

Table 2. Stimulation of tubulin polymerization by HeLa MAPs

HeLa tubulin, mg/ml	HeLa MAPs, mg/ml	Brain tubulin, mg/ml	Microtubule formation*	% protein sedimented†
0.5	—	—	−	7
—	0.15	—	−	3
0.55	0.08	—	+	46
0.53	0.15	—	+	73
—	0.15	0.7	+	84

We incubated samples at 37°C for 30 min to test for polymerization. HeLa tubulin, HeLa MAPs, and brain tubulin were purified by using DEAE ion exchange chromatography (see *Materials and Methods*). The brain DEAE-purified tubulin did not self-assemble significantly under these conditions as reported (5).

*Microtubule formation was assayed by electron microscopy of negatively stained samples.

†The extent of polymerization was determined by a quantitative sedimentation assay (28).

The results are shown in Table 2. Microtubules formed only in the samples containing MAPs, and a gel electrophoretic pattern of the MAP-induced polymerization product is shown in Fig. 4.

It should be noted that the MAP fraction used in this experiment was obtained from thrice-cycled HeLa microtubule protein, H_3P, and contained in addition to the HeLa MAPs described above, small amounts of material of molecular weights 100,000, 80,000, and 50,000, which are normally absent from four-times-cycled material. The 200,000–220,000- and 120,000–125,000-molecular weight components bound to and cosedimented with the tubules, whereas the other species remained in the supernatant in the sedimentation assays and failed to sediment with the polymer. Therefore, by the criterion of this reconstitution experiment, the HeLa MAPs that we have described above specifically associate with HeLa tubulin and effectively stimulate brain or HeLa microtubule polymerization, whereas the species of molecular weights 100,000, 80,000, and 50,000 are characterized by the same criteria as residual contaminants.

DISCUSSION

In this report we have demonstrated the purification of microtubule protein from a cultured cell line by using a modification of a method previously employed for brain tissue (4). The important features of the present method are (*i*) gentle lysis to avoid the introduction of inhibitory activity, (*ii*) very high protein concentration to insure that the tubulin concentration in the extract is significantly above the critical concentration for assembly, and (*iii*) the inclusion of EGTA and dithiothreitol. Under these conditions, the majority of tubulin in the HeLa extract was competent to polymerize without the addition of glycerol, microtubule seeds, or other exogenous polymerization stimulatory agents. The polymerization was reversible, occurred in good yield, and has been carried through four cycles of assembly–disassembly. Thus, the three criteria outlined in the introduction for an adequate assembly system have been met.

In such a system we may ask whether microtubule-associated proteins (MAPs) are present; but first, an important distinction must be made. We and others originally reported that high molecular weight proteins specifically associate with microtubules of brain tissue on the basis of copurification through multiple cycles of assembly and fixed stoichiometry relative to tubulin (4, 35, 36). We later showed (13) that, although many cellular proteins bind to microtubules, only a few proteins, namely the high molecular weight species, exhibit a high affinity for tubulin. Berkowitz *et al.* (14) have also characterized in detail the copurifying proteins of brain microtubules and have suggested a useful differentiation. They divide the proteins into two groups designated Q-MAPs or NQ-MAPs according to whether the proteins remain in constant quantitative ratio to tubulin during the purification cycles or become removed during purification, albeit inefficiently. The only Q-MAPs they found were the high molecular weight species we reported plus two low molecular weight species between 30,000 and 35,000. These latter species may be proteolytic fragments of the major brain MAP species, because in another study (34) we showed that peptides of that molecular weight, retaining the ability to bind to tubulin and stimulate polymerization, could be derived from this brain MAP by controlled digestion with trypsin. Other components including those referred to in the literature as tau factor (6) and tubulin assembly protein (11) were placed in the NQ-MAP group.

On the basis of quantitative copurification with tubulin through four cycles of polymerization and depolymerization,

the HeLa species of molecular weights 120,000–125,000 and 200,000–220,000 clearly belong to the Q-MAP class. In addition, these proteins satisfy two other criteria for the identification of MAPs; namely, stimulation of microtubule polymerization when added to pure tubulin and cosedimentation with tubulin in a reconstitution assay.

The HeLa MAP species share some properties with MAPs isolated from brain tissue. HeLa MAPs recognize and bind to brain tubulin as well as to HeLa tubulin. Like brain MAPs, they seem to exert their stimulatory effects by lowering the equilibrium monomer concentration required for polymerization, although detailed quantitative studies have not yet been carried out. The DEAE ion-exchange procedure used to separate brain tubulin and MAPs serves with slight modifications to separate HeLa tubulin and MAPs.

No other Q-MAPs have been conclusively demonstrated in cultured cells. Nagle *et al.* (18) have reported a putative MAP of molecular weight 49,000 in various cultured cells, and Weatherbee *et al.* (17) have described a 68,000-molecular weight species in HeLa cells. However, in both of these studies only two cycles of polymerization were carried out and hence no demonstration of quantitative copurification was made. Neither of these groups demonstrated that their putative MAPs possessed polymerization stimulatory activity or cosedimented with tubulin in a reconstitution assay. We observed a species of molecular weight slightly greater than 68,000 in HeLa extracts and once- and twice-cycled material, but the species failed to quantitatively copurify and was absent from thrice-cycled protein. A protein of molecular weight 50,000 was still detectable in thrice-cycled material, but it failed to cosediment with microtubules in reconstitution experiments. Thus, we were unable to determine whether these components were NQ-MAPs or weakly associating contaminants.

This study was supported by National Institutes of Health Grants GM 21963 and GM 25062 (to G.G.B.). J.C.B. is a National Institutes of Health Predoctoral Trainee (Grant 144L323).

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