IN VITRO POLYMERIZATION OF MICROTUBULES
FROM HeLa CELLS

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ABSTRACT
Although the purification of microtubules from brain by alternate cycles of polymerization and depolymerization in vitro has become routine, the application of this method to non-neural, cultured cells has been less successful. Previous investigations have suggested that it was necessary to use substrate-grown cells and 4 M glycerol to obtain microtubules from cultured cells. We have developed a method for preparing microtubules from HeLa cells in spinner cultures without the use of glycerol. Microtubules can be readily carried through two complete cycles of polymerization at 37°C and depolymerization at 4°C in vitro. The microtubules obtained are morphologically similar to brain microtubules in electron micrographs, and the tubulin subunits have mobilities similar to those of brain tubulins on polyacrylamide gels. Typical yields in the second polymerization pellet are about 1 mg protein/ml of packed cells or 2.5-3.0% of the total protein in the soluble cell extract. The major nontubulin protein present after two cycles of polymerization and depolymerization has an apparent mol wt of 68,000 daltons. If glycerol is used during polymerization, this band is virtually absent.

KEY WORDS microtubules · tubulin · HeLa cells · microtubule-associated proteins (MAP's) · high molecular weight microtubule-associated proteins (HMW's) · tau protein

Virtually all of the detailed information about microtubule accessory proteins currently available and most of what is known about factors that control microtubule polymerization in vitro has been derived from experiments with brain microtubules. The methods which were developed for the in vitro polymerization of microtubules from brain (25, 34) have been successfully extended to polymerization of microtubules from only a few other sources. These include bovine renal medullary tissue (1), bovine anterior pituitary (27), Drosophila embryos (12), human and porcine platelets (4, 5, 14), and Ehrlich ascites tumor cells (10).

Particular difficulty has been encountered in preparing microtubules from cultured cells. Early attempts to polymerize microtubules from extracts of cultured cells were unsuccessful (2, 22), and it was suggested that these extracts contained an inhibitory factor which prevented polymerization (3). More recently, however, microtubules have been prepared from a few cell lines, including C6 glial cells (19, 35), both normal and simian virus 40 (SV40) transformed 3T3 cells (18, 19, 32, 36), CHO cells (19), and neuroblastoma cells (19). In almost all of these reports, the cells were grown attached to a substrate, and 4 M glycerol was used during the polymerization stage of purification. It has been suggested that both of these conditions...
are important for success in preparing microtubules from cultured cells (18).

Here we report the successful preparation of microtubules from extracts of HeLa cells grown in suspension culture. These microtubules can be reversibly polymerized in vitro. As expected, they contain α- and β-tubulin, and they also contain a protein of about 68,000 daltons, which has not previously been reported to co-purify with cultured cell microtubules. We have found that it is not necessary to use glycerol in this procedure and that, in fact, if glycerol is used in the polymerization step of this method, the microtubules no longer contain the 68,000 dalton component.

MATERIALS AND METHODS

HeLa cells (Strain S-3) were grown in spinner bottles as described previously (13). About 2.5-3 liters of medium with cell densities of 5-7 x 10^6 cells/ml were routinely harvested by low-speed centrifugation. About 6.5-8.5 ml of packed cells is obtained this way. After collection, the cell pellet was washed once with about 2 vol of phosphate-buffered saline (PBS) containing 150 mM NaCl in 10 mM phosphate buffer (sodium salt) at pH 7.0 and once with about the same volume of 1 mM MgSO_4, 2 mM ethyleneglycolbis[β-aminoethyl ether]-N,N'-tetraacetic acid (EGTA), 0.1 mM GTP (type II-S, Sigma Chemical Co., St. Louis, Mo.), and 100 mM piperazine-N,N'-bis[2-ethane sulfonic acid] (PIPES) (Sigma Chemical Co.), pH 6.9 (PM buffer). The cells were pelleted from the second wash in conical graduated centrifuge tubes in an IEC model PR-1 refrigerated centrifuge (IEC Corporation, Austin, Texas). The packed cell volume was noted, and the cells were resuspended in an equal volume of cold PM buffer. Cells were then disrupted by sonication at 0°C with three treatments of 30 s each at 50 W using the standard microtip of a Sonifier cell disrupter (Model 185; Branson Sonic Power Co., Div. of Branson Ultrasone Corp., Danbury, Conn.). Cells were also usually then treated by five strokes in a glass Potter-Elvehjem homogenizer equipped with a Teflon pestle. The extract was then centrifuged for 30 min at 35,000 × gavg and 4°C. This and subsequent centrifugations were carried out in an IEC model PR-1 refrigerated centrifuge (IEC Corporation, Austin, Texas). The final supernate was then suspended in about one milliliter of PM buffer, and the cold incubation and centrifugation were repeated. The final supernate was then stored at -80°C in a Revco freezer (Revco, Inc., West Columbia, S.C.) until immediately before further work.

When glycerol was used, this procedure was modified as follows: The 35,000 × gavg supernate was mixed with an equal volume of cold 8 M glycerol in PM, and then GTP was added to 2 mM. After the incubation at 37°C, microtubules were collected by centrifugation at 100,000 × gavg for 30 min. Glycerol was also added in a similar manner to the second 37°C polymerization.

Chick brain microtubules were prepared as described previously (31). Chick brain microtubules were prepared using glycerol essentially according to the method described by Dentler et al. (9).

For gel filtration, Sepharose 4B (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N.J.) was used. Approx. 6 mg of purified microtubule protein in 1.65 ml of PM buffer was applied to a 1.5 x 26-cm (bed volume = 46 ml) column equilibrated in PM buffer at 4°C. Elution was continued with the same buffer, and 0.75-ml fractions were collected and analyzed.

Phosphocellulose (Whatman P11; H. Reeve Angel & Co., Inc., Clifton, N.J.) was precelled as described by Sloboda et al. (28). A 0.9 x 20-cm (bed volume = 13 ml) column was equilibrated with PM buffer at 4°C. Approx. 10.5 mg of purified microtubule protein in 2.5 ml of PM buffer was applied, and the column was washed with about 20 ml of PM buffer. Column flow rate during sample application and elution was approx. 0.1 ml/min. (about one-half column bed volume/h.). Adsorbed protein was eluted with a linear gradient of 0-0.5 M NaCl in PM (total volume = 50 ml). One-milliliter fractions were collected and analyzed.

DEAE-cellulose (Whatman DE-52; H. Reeve Angel & Co., Inc.) was equilibrated in PM buffer at 4°C. Approx. 8.5 mg of purified microtubule protein in 2.5 ml of PM buffer was applied to a 0.9 x 15-cm (bed volume = 10 ml) column, and the column was washed with about 20 ml of PM buffer. Adsorbed protein was eluted with a linear gradient of 0-0.5 M NaCl in PM (total volume = 50 ml). One-milliliter fractions were collected and analyzed.

Protein concentrations were routinely determined by the method of Lowry et al. (15) using bovine serum albumin (Sigma Chemical Co.) as a standard. NaDodSO_4/polyacrylamide gel electrophoresis was carried out according to the method of Neville (20), using the staining and destaining protocol of Fairbanks et al. (11). For electron microscopy, 0.01-ml portions of samples were mixed at 37°C with an equal volume of 1% glutaraldehyde in PM buffer (1 mM GTP). Carbon-coated 400-mesh grids were floated on this mixture for 30 s, rinsed by touching the grid five times to distilled water, floated on a drop of 2% uranyl acetate for 1 min, and dried by touching to filter paper. The negatively stained samples were examined in a Philips EM 301 electron microscope.
RESULTS

Isolation of HeLa Cell Microtubules

Despite earlier suggestions of difficulties encountered in polymerizing microtubules from cultured cells, particularly those grown in spinner cultures (18), we have found that microtubules can be prepared from suspension cultures of HeLa cells by methods that have been modified only slightly from those used previously to prepare microtubules from brain extracts (31). Two factors that have been found to be important are (a) maintenance of high protein concentration by use of small extraction volumes, and (b) disruption of cells by sonication.

The procedure described for the preparation of microtubules from HeLa cells is convenient and routine, having been carried out on 12 separate batches of cells without failure. Two unique features of the method, aside from the use of HeLa cells, are that cells from spinner cultures could be used and that the use of glycerol was avoided. Microtubules prepared by this method can be again polymerized after storage for at least 2 mo at -80°C. Isolated HeLa cell microtubules are morphologically similar to brain microtubules when examined by negative staining (Fig. 1), and the tubulin subunits of HeLa microtubules have mobilities similar to those of brain tubulin on NaDodSO4/polyacrylamide gels (Fig. 2b).

The use of spinner cultures for growing HeLa cells allows us to obtain relatively large numbers of cells conveniently and to prepare sufficient amounts of microtubules for biochemical characterizations. From a suspension culture containing 1.5-2 × 10^9 cells, representing a packed cell volume of 6.5-8.5 cm^3, we can usually isolate 3-5 mg of microtubule protein after two complete cycles of polymerization and depolymerization. The material obtained in the second polymerization pellet amounts to an average of 2.8% of the total soluble protein present in the supernates of crude cell extracts. This yield is comparable to that routinely obtained from chick brain extracts without the use of glycerol.

Fig. 2a shows NaDodSO4/polyacrylamide gels of each step of a typical purification procedure. It can be seen that, after two complete cycles of polymerization and depolymerization, a major component with a molecular weight somewhat larger than tubulin (Fig. 2a, small arrow) is present in these microtubules. A number of other protein species are also present in lesser amounts. Microtubules purified from chick brain by a similar procedure are shown as a comparison in Fig. 2b. It can be seen that for HeLa cell microtubules there are no high molecular weight components present in amounts comparable to those seen in chick brain preparations. Based on the molecular weight standards shown in Fig. 2b, the major nontubulin component of HeLa cell microtubules has a mol wt of about 68,000 daltons.

A previous report has suggested that the use of glycerol during the preparation of microtubules from porcine brain by in vitro polymerization decreased the amount of high molecular weight proteins found associated with these microtubules (23). We have found a similar effect of glycerol
on the composition of chick brain microtubules. In a typical preparation of chick brain microtubules after two complete cycles of polymerization and depolymerization without glycerol, we find that approx. 11% of the protein consists of two high molecular weight proteins (MAP-1 and MAP-2) and that approx. 7% consists of two proteins with mol wt of approx. 58,000 and 60,000 daltons which apparently correspond to the major proteins of the tau fraction of porcine brain microtubules described by Weingarten et al. (33) and Witman et al. (37). The lower of these two "tau" fraction bands is usually not clearly resolved from the upper tubulin band on heavily loaded gels. The remainder of the protein in these preparations consists of tubulin and a number of proteins present in trace amounts (J. A. Weatherbee, unpublished observations). A NaDodSO₄/polyacrylamide gel of such a preparation is shown in Fig. 3a. If glycerol is used during microtubule preparation, the composition of the microtubules obtained is greatly altered. Fig. 3b shows a gel of a preparation made using glycerol loaded with the same amount of protein as in Fig. 3a. It can be seen that the amount of high molecular weight protein is greatly decreased and that the 58,000-
microtubules might be a functional microtubule accessory protein, we were interested in determining whether this component had properties corresponding to those of the brain accessory proteins which have been previously described (17, 21, 29). Therefore we attempted to fractionate HeLa microtubules by methods which have been used to fractionate brain microtubules. These included gel filtration on Sepharose 4B, cation exchange chromatography on phosphocellulose (Whatman P11), and anion exchange chromatography on DEAE-cellulose (Whatman DE-52).

The results of gel filtration on Sepharose 4B are shown in Fig. 5. Tubulin and the 68,000-dalton component elute together in one included peak (Fig. 5a, and b). Even though some of the minor and 60,000-dalton proteins are no longer visible.

Because of this effect of glycerol on the composition of microtubules, we have avoided its use during the routine preparation of HeLa cell microtubules. For comparative purposes, however, we have twice prepared HeLa microtubules using 4 M glycerol in the polymerization steps of the procedure. Gels of one of these preparations are shown in Fig. 4. It can be seen that the protein with a mol wt of 68,000 daltons, which was a major component of the preparation made without glycerol, is no longer seen. Only trace amounts of any proteins besides tubulin are present.

**Fractionation of HeLa Cell Microtubules**

Because of the possibility that the 68,000-dalton protein which co-purifies with tubulin in HeLa microtubules might be a functional microtubule accessory protein, we were interested in determining whether this component had properties corresponding to those of the brain accessory proteins which have been previously described (17, 21, 29). Therefore we attempted to fractionate HeLa microtubules by methods which have been used to fractionate brain microtubules. These included gel filtration on Sepharose 4B, cation exchange chromatography on phosphocellulose (Whatman P11), and anion exchange chromatography on DEAE-cellulose (Whatman DE-52).

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components in the preparation appear to be partially resolved from the tubulin peak, the 68,000-dalton component seems to elute in a constant ratio with tubulin across the peak. This result suggests that the two components are associated. There is no evidence from the Lowry protein assays or OD₂₅₀ (not shown) for the presence in the region of the void volume (around fraction 16 on this column) of any major concentration of large aggregates of the type characteristically found in brain microtubule preparations in the cold ("rings" [28]). For example, on a column of this type, we have found...
that for chick brain microtubules there was a peak near the void volume of the column which contained rings and consisted mainly of high molecular weight MAP's and tubulin (see, for example, Weatherbee et al [31]).

The phosphocellulose column (a cation exchanger) provided only a partial separation of tubulin from the 68,000-dalton component (Fig. 6). The peak of material which passed through the column without being adsorbed contained both

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**Figure 6** Phosphocellulose fractionation of HeLa microtubules. (a) Phosphocellulose (Whatman P-11) (bed volume = 13 ml) was equilibrated with PM buffer containing 0.1 mM GTP. A sample of 2 x polymerized HeLa cell microtubules was applied in the same buffer (volume = 2.5 ml), and elution with this buffer was continued (fractions = 1.0 ml). At fraction no. 20, elution was begun with a linear gradient of NaCl (0-0.5 M) in PM buffer (0.1 mM GTP). 0.05-ml aliquots of each sample were removed, diluted with 0.15 ml of H2O, and assayed for protein by the method of Lowry et al. (15). Fractions across each of two peaks (I and II) were analyzed by NaDodSO4/polyacrylamide gel electrophoresis. (b) NaDodSO4/polyacrylamide gels of column fractions indicated in Fig. 6a. Electrophoresis conditions were as described in Fig. 2. 0.06 ml or a maximum of 50 µg of protein was taken from each fraction across peaks I and II. (Column a) Unfractionated HeLa microtubules. (Column b) Chick brain microtubules. Large arrows indicate α- and β-tubulin. Small arrow indicates the 68,000-dalton protein.
tubulin and most of the 68,000-dalton component (Peak I, Fig. 6). The remainder of the 68,000-dalton component and all of the other minor components adsorbed to the column and were eluted as one peak at about the middle of the 0–0.5 M NaCl gradient (Peak II, Fig. 6). The incomplete separation may have been a result of the particular batch of phosphocellulose used.

The DEAE-cellulose column (an anion exchanger) gave a cleaner separation (Fig. 7). The

![Graph](image)

**Figure 7** DEAE-cellulose fractionation of HeLa microtubules. (a) DEAE-cellulose (Whatman DE-52) (bed volume = 10 ml) was equilibrated with PM buffer (0.1 mM GTP). A sample of 2 × polymerized HeLa cell microtubules was applied in the same buffer (volume = 2.4 ml) and elution with this buffer was continued (fractions = 1.0 ml). At fraction no. 20, elution was begun with a linear gradient of NaCl (0–0.5 M) in PM buffer (0.1 mM GTP). 0.05-ml aliquots of each fraction were removed, diluted with 0.15 ml of H2O, and assayed for protein by the method of Lowry et al. (15). Fractions across each of three peaks (I, II, and III) were analyzed by NaDodSO4/polyacrylamide gel electrophoresis. (b) NaDodSO4/polyacrylamide gels of column fractions indicated in Fig. 7a. Electrophoresis conditions were as described in Fig. 2. 0.06 ml or a maximum of 50 μg of protein was applied to each gel. (Column a) Unfractionated HeLa microtubules. (Column b) Chick brain microtubules. (Column c) Standards: (bottom to top) actin, bovine serum albumin, β-galactosidase. Lines indicate the three standards. Large arrows indicate α- and β-tubulin. Small arrow indicates the 68,000-dalton protein.
68,000 component and most of the other minor components passed through the column without being adsorbed (Peak I, Fig. 7). The salt gradient (0–0.5 M NaCl) eluted first a peak containing a few of the other minor components (Peak II, Fig. 7) and finally a single peak of clean tubulin (Peak III, Fig. 7). The DEAE-cellulose results suggest that the 68,000-dalton component is likely to have a positive charge at neutral pH, as do almost all of the brain microtubule accessory proteins studied so far (6, 7, 17, 28). If this is true, then the reason why this protein is not adsorbed by phosphocellulose may be because it is tightly complexed to tubulin.

In preliminary experiments, using DEAE-cellulose columns and conditions similar to those just described with the exception that 2 mM dithioerythritol (DTE) was included in our usual buffer, we found that the protein fraction of HeLa microtubules that is not adsorbed to the column (essentially similar to the Peak I fraction of Fig. 7) can promote the formation of microtubules when added to purified HeLa tubulin (similar to Peak III of Fig. 7). No microtubules are formed from the purified tubulin alone under these conditions at the same concentration of tubulin (1 mg/ml). Quantitatively, greater amounts of micotubules are found on electron microscope grids of negatively stained preparations when increasing amounts (from 0.17 to 1.4 mg protein/ml) of the fraction are added to tubulin (maintained at 1 mg/ml). The major component of the nonadsorbed fraction is the 68,000 protein, but a number of other proteins are also present. We have also found that samples containing essentially only tubulin and the 68,000-dalton component (the nonadsorbed peaks from phosphocellulose columns, e.g., Peak 1, Fig. 6), will polymerize in our usual polymerization buffer at concentrations below 1.5 mg protein/ml. Because of these observed properties of the 68,000-dalton component, we believe that this component represents a microtubule accessory protein of the same type as, but clearly distinct from, the tau and high molecular weight proteins of brain. We also suggest that the use of glycerol in the polymerization steps of the microtubule purification procedure displaces this accessory protein and allows polymerization to occur in the absence of the 68,000-dalton component. This explains why other investigators, all of whom use glycerol, rarely find substantial amounts of any protein besides tubulin in their microtubule preparations from cultured cells (see, e.g., Weber et al [32]).

DISCUSSION
Currently, there is considerable controversy concerning the type, distribution, and relative importance of various microtubule accessory proteins. The best characterized accessory proteins are those found associated with mammalian brain microtubules. Two major categories of accessory protein from pig and calf brain have been described and more or less characterized. The first consists of a group of a varying number of high molecular weight (greater than 200,000 daltons) proteins designated microtubule-associated proteins (MAP's) (9, 28, 29) or high molecular weight (HMW) proteins (16, 17). The second group of accessory proteins consists of a number of closely related components with molecular weights in the range of 55–62,000 daltons which have been called the tau fraction (6, 7, 33). At present there is disagreement with respect to the relative importance and abundance of the HMW/MAP fraction compared to the tau fraction. It is not yet clear whether these discrepancies are due to species differences, differences in isolation conditions, or other factors.

Whatever the relative importance of these two types of proteins, the studies of their properties cited above show that either fraction can act as an effective stimulator of the in vitro polymerization of tubulin to form microtubules. It remains to be demonstrated that these accessory proteins perform a similar function in the mediation of tubulin polymerization in vivo. Recent observations which show that fluorescein-conjugated antiserum to pig brain tau protein stains a Colcemid-sensitive network in interphase mouse embryo fibroblasts and mitotic spindles in dividing cells (8) suggest at least some in vivo function for tau-type accessory proteins. Similar observations have been made that antibodies prepared against rat brain HMW proteins stain a filamentous array in N115 neuroblastoma cells and 3T3 fibroblasts (26).

Accessory proteins associated with microtubules from nonneural sources have not been so well characterized as the tau and HMW/MAP fractions of brain, and it is not clear how closely accessory proteins from different sources are related. Microtubules from C6 glial cells (35), platelets (4, 5, 14), and bovine anterior pituitary extracts (27) have been reported to contain variable amounts (5–10%) of high molecular weight components which may correspond to the HMW/MAP proteins of brain. The situation is less clear for tau-like proteins. Microtubules isolated from Ehrlich ascites tumor cells (10), from renal med-
ullary tissue (1), and from bovine anterior pituitary (27) are reported to have some proteins of intermediate molecular weight associated with tubulin, but it is not yet known how closely they correspond to mammalian brain tau protein.

In a study which appeared as this manuscript was being completed, the preparation of microtubules from several cell lines (including neuroblastoma, C6 glioma, and Chinese hamster ovary (CHO) cells) using glycerol was reported (19). In most cases, purification of microtubules was carried through only one cycle of polymerization and depolymerization, but results were presented for neuroblastoma microtubules which were carried through two complete cycles. A major nontubulin protein with an apparent mol wt of about 49,000 daltons was found in these preparations. These results do not agree with our results with HeLa cell preparations. It is important to determine whether these differences are due to real differences in microtubules from various sources or differences arising from variations in techniques of purification. A complication in comparing these results with ours arises from the fact that a protein with a mol wt of 49,000 daltons would probably not be resolved from β-tubulin in our gel system. Nevertheless, we do not find a major nontubulin protein of this size among the proteins separated from tubulin on either DEAE-cellulose or phosphocellulose columns.

So far, we have also not found high molecular weight proteins present in any substantial amount in our HeLa cell microtubule preparations, despite the suggestions that at least some types of cultured cells contain such proteins (26, 35). It may be that HeLa cells do not contain proteins of this type. Alternatively, these proteins may be present in vivo, but may be destroyed during the microtubule purification procedure. This suggestion is not implausible in view of the known sensitivity of brain accessory proteins to protease digestion (30, 31) and of the increased protease activity level of a transformed cell line such as HeLa (24). This possibility is currently being investigated.

Although no high molecular weight accessory proteins are present, we believe that the evidence which we have presented suggests that the 68,000-dalton protein which co-purifies with tubulin from HeLa cells is an accessory protein with properties similar to those of the microtubule accessory proteins of brain. The molecular weight of this protein suggests a superficial resemblance to tau. However, this protein clearly does not comigrate with chick brain tau and does not show the microheterogeneity characteristic of brain tau (6, 7). Future work will be concerned with verification of the role of this protein as a functional microtubule accessory protein, additional characterization of this protein, a study of the possible relation of this protein to microtubule accessory proteins from other sources, and the in vivo function of this protein.

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