

Microtubule-associated Proteins of HeLa Cells: Heat Stability of the 200,000 mol wt HeLa MAPs and Detection of the Presence of MAP-2 in HeLa Cell Extracts and Cycled Microtubules

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ABSTRACT One of the major groups of microtubule-associated proteins (MAPs) found associated with the microtubules isolated from HeLa cells has a molecular weight of just over 200,000. Previous work has demonstrated that these HeLa MAPs are similar in several properties to MAP-2, one of the major MAPs of mammalian neural microtubules, although the two types of proteins are immunologically distinct. The 200,000 mol wt HeLa MAPs have now been found to remain soluble after incubation in a boiling water bath and to retain the ability to promote tubulin polymerization after this treatment, two unusual properties also shown by neural MAP-2. This property of heat stability has allowed the development of a simplified procedure for purification of the 200,000 HeLa MAPs and has provided a means for detection of these proteins, even in crude cell extracts. These studies have also led to the detection of a protein in crude extracts of HeLa cells and in cycled HeLa microtubules which has been identified as MAP-2 on the basis of (a) comigration with calf brain MAP-2 on SDS PAGE, (b) presence in purified microtubules, (c) heat stability, and (d) reaction with two types of antibodies prepared against neural high molecular weight-MAPs, one of these a monoclonal antibody against hog brain MAP-2. MAP-2, although present in HeLa cells, is at all stages of microtubule purification a relatively minor component in comparison to the 200,000 HeLa MAP's.

Some of the microtubule-associated proteins (MAPs) found associated with tubulin in the microtubules polymerized *in vitro* from neural sources have been extensively studied. Two major groups of brain MAPs have been distinguished and investigated. One group of proteins, with molecular weights in the range of 55,000–62,000, has been designated “tau” (8, 9, 46). The second group is of higher molecular weight (> 250,000) and has been designated MAP (15, 40) or HMW (high molecular weight) proteins (27, 28). The major component of this second group of proteins usually migrates as a pair of closely spaced bands on SDS polyacrylamide gels of microtu-

bules prepared from mammalian brain. The components of this pair have been designated collectively as MAP-2 (15, 23, 40), or individually as HMW-1 and HMW-2 (27). An additional component of the HMW fraction, usually present in lower amounts than MAP-2 and having a higher subunit molecular weight than MAP-2, is usually designated MAP-1 (15, 23). Both MAP-2 and the tau proteins have been shown to promote the net polymerization of tubulin *in vitro* (8, 9, 28, 40).

The MAPs of nonneural cells have, in most cases, not been characterized nearly so well, and the question of whether

nonneural cells contain proteins corresponding to the brain MAPs is currently controversial. Several studies using immunofluorescence microscopy have reported that antibodies prepared against the total tau fraction (11, 12, 13) or the total HMW-MAP fraction (11, 12, 14, 36, 37) react with fibrous networks which are apparently microtubular in a variety of nonneural cell types. Also using immunofluorescence microscopy, reactions with antibodies against purified MAP-2 have been shown for the microtubules of ovarian granulosa cells (38) and for the marginal band of newt erythrocytes (39). On the other hand, two other laboratories, one using an antibody which reacts selectively with MAP-2 (32), and the other using a monoclonal antibody against hog brain MAP-2 (21), observed reactions with cells of primary brain explants but not with a variety of nonneural cell types.

Conflicting results have also been obtained from analysis of the *in vitro* polymerization of microtubules from nonneural sources. Because of difficulties in obtaining sufficient amounts of material from these sources and because microtubules from some nonneural cell types have not yet been repeatedly cycled *in vitro*, several investigators have copolymerized cell extracts with brain microtubules or tubulin and determined the non-tubulin proteins which are found in microtubules after assembly-disassembly cycles. In several studies of this type, no proteins were found which corresponded to the HMW-MAPs or major tau proteins (24, 35, 41). On the other hand, one such study found proteins on SDS polyacrylamide gels apparently corresponding to both types of brain MAPs in extracts of simian virus 40-transformed 3T3 cells (10).

In a few cases, it has been possible to prepare microtubules from nonneural sources by reversible polymerization *in vitro*. Some studies of this type (16, 29, 47, 48) have used glycerol to promote tubulin polymerization, a procedure which hinders the identification of MAPs because the presence of glycerol lowers the concentration of proteins which copolymerize with tubulin (17, 34, 44). In a very few cases, microtubules have been prepared from cultured cells without glycerol (3, 17, 33, 44, 45). In no case, with or without glycerol, have MAPs been found which correspond to the major brain MAPs. In one case, however, it has been suggested that proteins in the molecular weight range of the HMW-MAPs of brain are present in a first polymerization pellet, but are lost at the depolymerization step, possibly by association with intermediate filaments (33).

The best-characterized nonneural microtubules have been isolated from HeLa cells. HeLa microtubules purified by repeated assembly-disassembly cycles contain, in addition to tubulin, major protein components with molecular weights of ~200,000 (201,000–206,000 [45] or 210,000 [3]) and ~100,000 (97,000–104,000, 114,000 [45] or 125,000 [3]), plus some minor components (68,000 [45], 255,000 [3, 4, 6]). The major HeLa MAP species have been shown to promote tubulin polymerization *in vitro* (6, 45) and have been localized intracellularly on microtubules using immunofluorescence microscopy (4). The 200,000 mol wt MAPs have also been found to be present in a variety of other human and primate cell types (5). The 200,000 mol wt HeLa MAPs have been shown to resemble neural MAP-2 in several properties including rather high subunit molecular weight, ability to promote tubulin polymerization, high asymmetry, and high sensitivity to protease digestion (6, 45). HeLa 200,000 mol wt MAPs and neural MAP-2 are, however, antigenically distinct, suggesting no direct relationship between the two types of proteins (4, 32, and results presented here).

Here we report that the HeLa 200,000 mol wt MAPs are heat stable, another property similar to neural MAP-2. This property has allowed the development of a simplified procedure for the purification of these proteins and has led to the development of a method for the detection of these proteins even in crude cell extracts. Additionally, we have now found that a protein identified as MAP-2 is indeed present in small but readily detectable amounts both in crude HeLa cell extracts and in cycled HeLa microtubules. The criteria for identification of this protein as MAP-2 are (a) comigration with calf brain MAP-2 on SDS polyacrylamide gels, (b) presence in cycled microtubules, (c) heat stability, and (d) reaction with two types of antibodies, one a monoclonal antibody against hog brain MAP-2.

MATERIALS AND METHODS

Cell Growth and Preparation and Fractionation of Microtubules

HeLa cells (strain S-3) were grown in spinner bottles as described previously (20). Two- and four-cycle HeLa microtubules and two-cycle calf brain microtubules were purified by *in vitro* assembly-disassembly cycles as described previously in detail (44, 45).

MAP-2 was prepared from two-cycle calf brain microtubules by the heat-treatment method of Fellous et al. (19) as modified by Kim et al. (23). A similar procedure was used to purify the 201,000–206,000 MAPs from two-cycle HeLa microtubules. HeLa microtubule samples which had been stored as second depolymerization supernates at -80°C were thawed rapidly and 3M NaCl was added to a final concentration of 0.75 M. Samples were then placed in a boiling water bath for 5 min. After this, samples were cooled on ice and centrifuged at 40,000 g_{max} for 30 min at 4°C . Supernates were carefully removed and applied to columns of Sepharose 4-B (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) equilibrated in polymerization buffer (PM) (1 mM MgSO_4 , 2 mM EGTA, 2 mM dithioerythritol (DTE), 0.1 mM GTP [type II-S, Sigma Chemical Co., St. Louis, Mo.], 100 KIU/ml Trasylol [FBA Pharmaceuticals, New York, N. Y.], 100 mM PIPES, pH 6.9) containing 0.75 M NaCl. Samples across the first peak to emerge from the column were pooled and simultaneously concentrated and dialyzed against PM buffer by pressure dialysis. Purified HeLa tubulin was prepared by a combination of phosphocellulose and DEAE-cellulose column chromatography (PC/DE tubulin) as described previously (45). The ability of heat-treated 201,000–206,000 mol wt HeLa MAPs to promote polymerization of purified HeLa tubulin was tested by the standard reconstitution assay also described previously (45).

The fractionation by heat treatment of samples taken at various stages during the course of a HeLa microtubules preparation was essentially as described above. At the appropriate steps, 0.9-ml samples were removed, rapidly frozen in dry ice, and stored at -80°C until further processing. After storage, samples were rapidly thawed and 0.3 ml of 3 M NaCl in PM buffer was added to each (final concentration, 0.75 M NaCl). Samples were then heated and centrifuged as described above.

Protein concentrations were routinely determined by the method of Lowry et al. (26) using bovine serum albumin (BSA) as a standard. Negative staining and electron microscopy were carried out according to a previously described protocol (45) and samples were examined in a Philips EM 301 electron microscope.

SDS PAGE and Antibody Incubations

SDS PAGE was carried out according to the method of Neville (30) using running gels of 5% polyacrylamide. For permanent staining with Coomassie Brilliant Blue R, the staining and destaining protocol of Fairbanks et al. (18) was used. Densitometry of gels was carried out using a 570-nm filter in a Helena Quick Scan, jr. (Helena Laboratories, Beaumont, Tex.) modified from the standard apparatus so as to have smaller slits and a linear optical bench. The following proteins were used as electrophoresis standards: rabbit striated muscle myosin (22), pig α -actinin (generously provided by Dr. Darrell Goll, University of Arizona), rabbit striated muscle actin [42], and BSA (Sigma Chemical Co.).

Detection of specific antigens on SDS polyacrylamide gels was carried out using a modification of the methods described by Burridge (7) and Adair et al. (1). Duplicate samples were run on each half of 5% polyacrylamide slab gels. After electrophoresis, the dye fronts and upper right corners of the gels were marked with India ink. To facilitate cutting of lanes from the gels, protein bands were reversibly stained with Coomassie Blue G250 using the protocol of Blakesley

and Boezi (2). Bands were visible following 30–60 min of incubation. After this, one lane of each duplicate set was permanently stained with Coomassie Brilliant Blue R. The other lane of each set was fixed and completely destained in 25% isopropanol, 10% acetic acid (overnight with at least one change) in water. Gels were then washed in phosphate-buffered saline plus azide (PBS-azide) (15 mM KPi, 0.9% NaCl, 0.02% sodium azide, pH 7.0) until the pH of the wash was 7.0 (usually four washes of 1 h each). Gels were removed from the wash, blotted with a Kimwipe, and covered with a piece of filter paper cut to the exact size of the gel. The filter paper was flooded with antibody diluted appropriately in PBS-azide containing 10% normal goat or rabbit serum or 5 mg/ml BSA as carrier. After overnight incubation in a humidified chamber, gels were extensively washed with PBS-azide (2 d with seven or eight changes). Gels were again blotted and covered with filter paper and were incubated with ¹²⁵I-Protein A (500,000 cpm/ml) diluted in PBS-azide containing 0.25% gelatin as carrier. After this incubation, gels were again washed extensively with PBS-azide and dried. Autoradiographs were prepared using preflashed Kodak X-Omat R film and a DuPont Cronex Lightning-plus ZA intensifier screen (DuPont Instruments, S & P Div., Wilmington, Del.).

Two antibodies were tested on the SDS polyacrylamide gels of HeLa extracts and microtubules. The first was an antibody prepared in rabbit against the whole HMW-MAP fraction of rat brain microtubules. For overlay, the antibody was diluted 1:30 in PBS-azide containing 10% normal rabbit serum. The preparation and characterization of this antibody has been described previously (36, 37). The second antibody tested was a monoclonal antibody prepared against hog brain MAP-2. For overlay, this antibody (from ascites fluid) was diluted 1:50 in PBS-azide containing 5 mg/ml BSA. The preparation and characterization of this antibody have also been described previously (21). Protein A from *Staphylococcus aureus* (Pharmacia Fine Chemicals), iodinated using chloramine T, was the gracious gift of Dr. Harriet Robinson, Worcester Foundation for Experimental Biology.

Indirect Immunofluorescence Microscopy

HeLa cells grown on 12-mm cover slips were fixed with absolute methanol (−20°C, 4–6 min) and rinsed three times in PBS (pH 6.95) at 37°C. Cover slips were overlaid with the rabbit antiserum to the whole HMW-MAP fraction of brain microtubules (36, 37) at a 1:10 dilution for 30–40 min, rinsed with PBS, overlaid with fluorescein-labeled goat anti-rabbit IgG antibody (Miles Laboratories, Elkhart, Ind.) for 30–40 min, and rinsed again with PBS to remove excess second antibody. The cover slips were inverted onto drops of 50% glycerol on slides, and viewed under a Zeiss Photomicroscope III equipped with epifluorescence optics. Photographs were taken with the Zeiss built-in camera and Tri-X Pan 135 film.

RESULTS

We reported previously the purification of the 201,000–206,000 mol wt MAPs from HeLa microtubules and showed that these proteins can promote polymerization of purified HeLa tubulin (45). This purification procedure involved fractionation of four-cycle HeLa microtubules on DEAE-cellulose columns. It was necessary in this procedure to fractionate microtubules carried through four cycles, with the losses resulting from repeated *in vitro* cycling, because the 201,000–206,000 mol wt proteins were selectively lost from column fractions prepared from two-cycle microtubules, apparently as the result of the action of a protease present in the preparations at this stage of purification (45).

An alternate method of purification of the 201,000–206,000 mol wt proteins has now been developed which avoids both the need for starting with four-cycle microtubules and the losses due to proteolysis in less pure preparations. This procedure takes advantage of the observation that the 201,000–206,000 mol wt proteins, like MAP-2 of mammalian neural microtubules, show the property of unusual thermal stability. Fig. 1 shows the enrichment for the 201,000–206,000 mol wt proteins achieved by heating two-cycle HeLa microtubules in a boiling water bath followed by centrifugation. Lane 1 shows a gel of unfractionated two-cycle HeLa microtubules. The second lane shows a gel of the proteins which precipitate after heating. The proteins which remain soluble after heating are

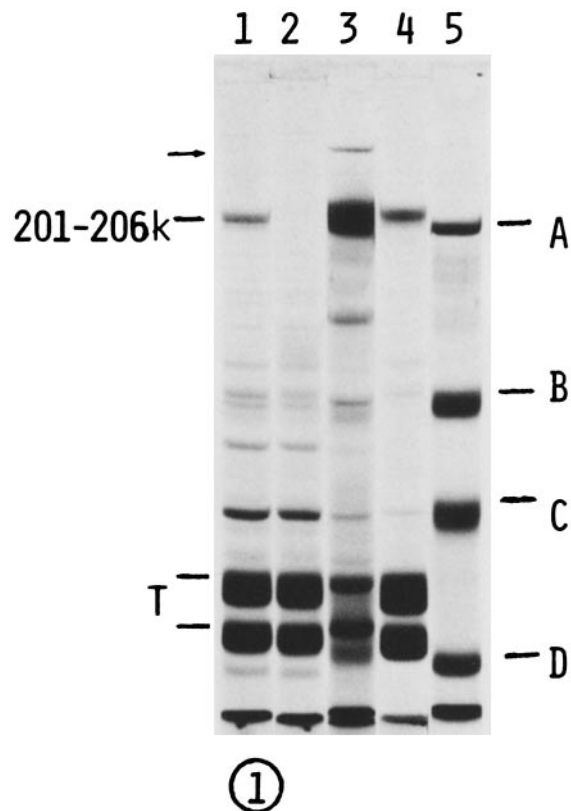


FIGURE 1 Heat treatment of HeLa microtubules. (Lane 1) Two-cycle HeLa microtubules. (Lane 2) Pellet obtained from centrifugation of two-cycle microtubules after treatment for 5 min in a boiling water bath (see text for details). (Lane 3) Supernate from centrifugation of heated two-cycle HeLa microtubules. (Lane 4) Four-cycle HeLa microtubules. (Lane 5) Molecular weight standards: (A) Myosin heavy chain (200,000); (B) α -actinin (100,000), (C) bovine serum albumin (68,000), (D) actin (42,000). Gels 1–4 were loaded with 50 μ g of protein each. Arrow indicates level of protein which comigrates with MAP2. T indicates tubulin bands.

shown in lane 3. It can be seen that the 201,000–206,000 proteins are present entirely in the centrifugation supernate and represent the most prominent component of this fraction. Supernatant fractions also always contain variable amounts of proteins which approximately comigrate with tubulin. It is not now clear whether these bands represent specific heat-stable proteins or are contaminants resulting from incomplete sedimentation or disturbance of pellets. Similar bands have been reported present in samples prepared in a similar manner from two-cycle calf brain microtubules (23). In that case, it was concluded, on the basis of two-dimensional gel electrophoresis, that the proteins were not tubulins, but no further characterization was reported.

Fig. 2 shows the purification of the 201,000–206,000 mol wt proteins achieved by chromatography of the supernates from heated two-cycle HeLa microtubules on Sepharose 4-B columns. Fractions across the first peak to emerge from the column were pooled and simultaneously concentrated and dialyzed (Fig. 2A, lane 2). The 201,000–206,000 mol wt protein fraction prepared in this way is not contaminated by the 68,000 mol wt protein found in fractions prepared by DEAE-cellulose chromatography of four-cycle microtubules (45). The 201,000–206,000 mol wt proteins prepared by heating and gel filtration retain the ability to promote the polymerization of purified

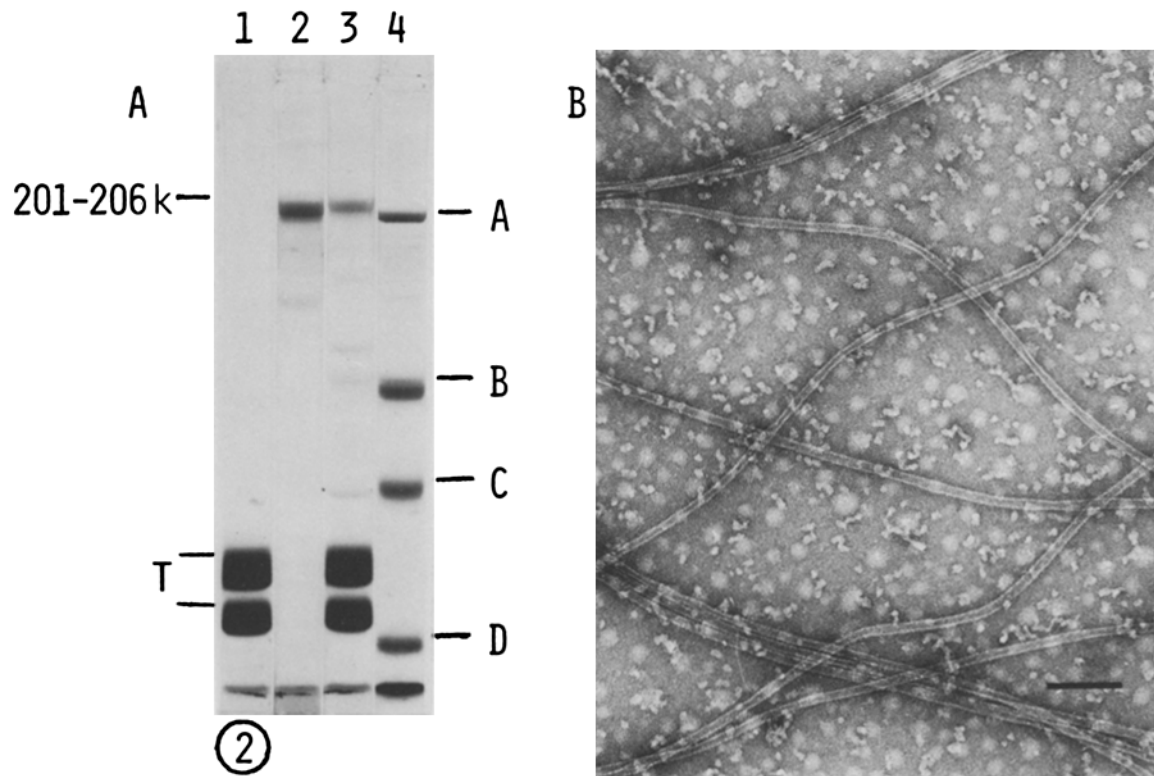


FIGURE 2 Promotion of tubulin polymerization by the 201,000–206,000 mol wt HeLa MAPs purified by heat treatment and gel filtration. (A, lane 1) HeLa tubulin purified from two-cycle microtubules by phosphocellulose and DEAE-cellulose column chromatography (PC/DE tubulin) (50 μ g). (Lane 2) 201,000–206,000 mol wt HeLa MAPs purified by heat treatment and gel filtration (see text) (10 μ g). (Lane 3) Four-cycle HeLa microtubules (50 μ g). (Lane 4) Molecular weight standards A–D as indicated in Fig. 1. (B) Electron micrograph of negatively stained microtubules polymerized by combining 201,000–206,000 mol wt proteins shown in lane 2 (final concentration, 0.22 mg/ml) with purified HeLa tubulin shown in lane 1 (final concentration, 1.0 mg/ml). Bar, 0.2 μ m. \times 45,000.

HeLa tubulin (Fig. 2A, lane 1) into apparently normal microtubules (Fig. 2B).

An unexpected result was the appearance in the supernates of heated two-cycle HeLa microtubules of a protein of higher molecular weight than the 201,000–206,000 mol wt HeLa MAPs (Fig. 1, lane 3 [arrow]). This protein was not detectable in unfractionated two-cycle microtubules (Fig. 1, lane 1). Since heating and centrifugation removes \sim 92% of the total protein from two-cycle HeLa microtubule preparations, an amplification factor of \sim 12 is produced for those proteins which remain soluble when the supernates of heated samples are run at the same total protein load per gel as unheated microtubules. This allows visualization of minor heat-stable components. This protein approximately comigrates on SDS polyacrylamide gels with MAP-2 from mammalian brain microtubules (see below). This observation, coupled with the presence of this protein in cycled microtubules (this protein has also been detected in three-cycle HeLa microtubules [not shown]) and the observation that this protein was heat-stable, an extremely unusual property for a protein with so high a subunit molecular weight,

To investigate this possibility, the distribution of this protein during the earlier stages of microtubule polymerization and depolymerization was followed to see if this HMW protein cofractionated with microtubules. We were also interested in developing a method for detecting the presence of the 201,000–206,000 proteins in crude cell extracts. Since both of these types of proteins are heat stable, it seemed that examination of supernates of heated samples would provide a method for accomplishing both of these aims. A procedure of this sort was recently used by Vallee (43) to detect the presence of MAP-2

in crude extracts of brain tissue and to demonstrate that MAP-2 is phosphorylated *in vivo* in brain.

The results of one such experiment are shown in Fig. 3. In this experiment, samples were taken at each step of a microtubule preparation, from sonication through one complete cycle of polymerization and depolymerization. The supernate from the second depolymerization (two-cycle microtubules) was also analyzed. Each sample was heated and the supernates were examined for the presence of the 201,000–206,000 mol wt proteins and the protein of high molecular weight. Both of these proteins were easily detectable even at the earliest stages of purification. The 201,000–206,000 mol wt proteins are almost completely released into the low speed supernate after sonication (Fig. 3, heat-treated supernates, lane 2). These proteins are sedimented after polymerization (lane 3), and largely released into the supernate following depolymerization (lane 6), although there is some loss of these proteins in the depolymerization pellet (lane 5). By the second depolymerization, these proteins are a major component of the heat-soluble fraction (lane 7). Generally, this distribution is what would be expected for a MAP.

Surprisingly, there are a number of very high molecular weight proteins ($>$ 250,000) present in the heat-stable fractions at the early stages of microtubule purification. Of these, however, only the protein which comigrates with MAP-2 is detectable in two-cycle microtubules (lane 7). This protein is also solubilized by sonication (Fig. 3, heat-treated supernates, lane 2), and almost completely sedimented following microtubule polymerization (lane 3). Unlike the 201,000–206,000 mol wt proteins, however, a large proportion of this protein remains

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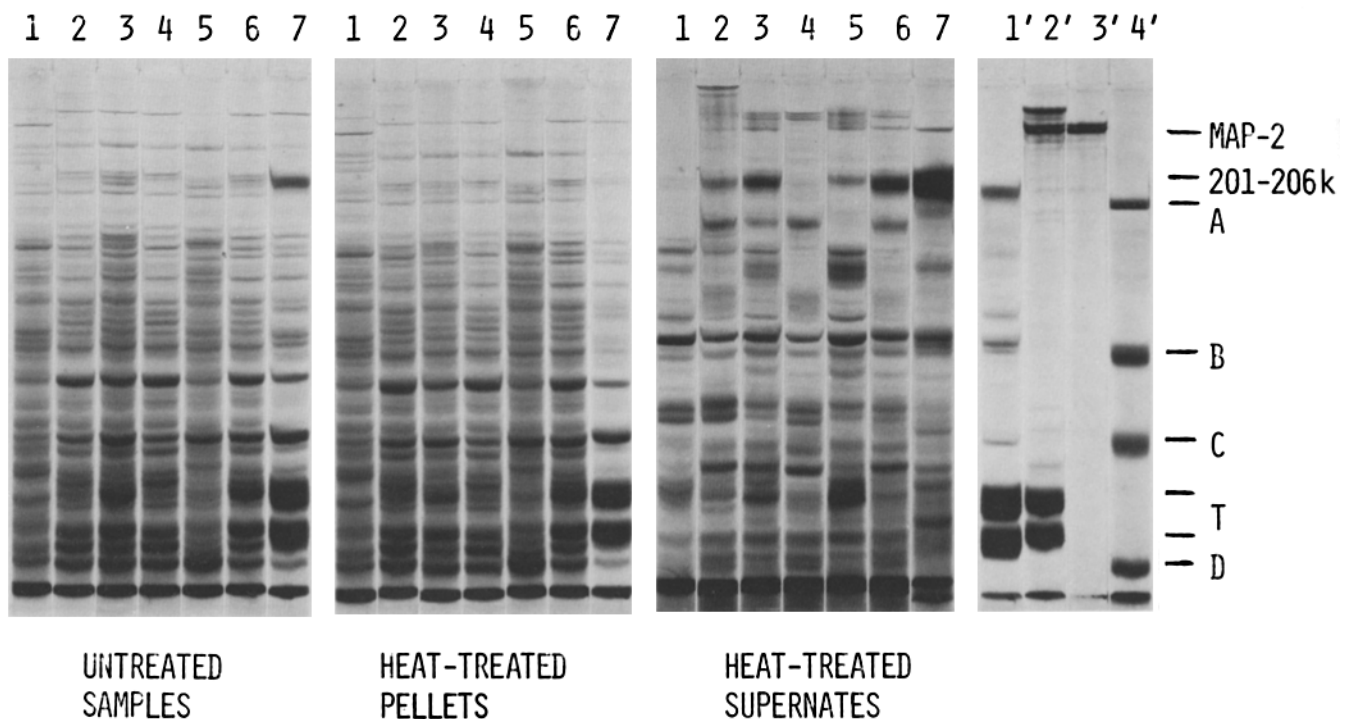


FIGURE 3 Fractionation by heat treatment of samples taken during the course of purification of HeLa microtubules by polymerization-depolymerization cycles. The group of gels at the far left is the untreated samples. The next group of gels represents the pellets obtained from centrifugation of the samples following heating. The third group of gels represents the supernates from centrifugation of the heated samples. Each gel was loaded with 50 μg of protein. (Lane 1) 35,000 g_{max} pellet of sonicated cells. (Lane 2) 35,000 g_{max} supernate of sonicated cells. (Lane 3) First polymerization pellet. (Lane 4) First polymerization supernate. (Lane 5) First depolymerization pellet. (Lane 6) First depolymerization supernate. (Lane 7) Second depolymerization supernate (two-cycle microtubules). At the far right are gels of reference proteins. (Lane 1') Four-cycle HeLa microtubules (50 μg). (Lane 2') Two-cycle calf brain microtubules (50 μg). (Lane 3') MAP-2 purified from two-cycle calf brain microtubules by heating and gel filtration (2.5 μg). (Lane 4') Molecular weight standards A–D as indicated in Fig. 1. T indicates tubulin bands.

sedimentable following depolymerization (lanes 5 and 6). Scans of gels at the depolymerization step indicate that about two-thirds of the total of this protein is sedimented and about one-third is present in the supernate. This loss is not repeated at the second depolymerization (not shown), and this protein is clearly detectable after two complete cycles of assembly-disassembly (lane 7). Again, with the exception of the high loss at the first depolymerization step, this distribution is generally what would be predicted for a MAP.

Stronger evidence that this HMW protein is indeed MAP-2 is provided by reactions with two types of antibodies. The first antibody was prepared against the total HMW fraction of rat brain microtubules (36, 37). Fig. 4A shows the reaction of this antibody with two-cycle calf brain microtubules. The antibody reacts with both MAP-1 and MAP-2 and with other minor bands which probably represent fragments of these higher molecular weight components. Fig. 4B shows the reaction of this antibody with the supernates of heated HeLa samples taken during a microtubule preparation, starting with the supernate from centrifugation after sonication and continuing through two cycles of assembly-disassembly (corresponding to lanes 2–7 of the heat-treated supernates shown in Fig. 3). It can be seen that this antibody reacts specifically with the heat-stable protein in these samples which comigrates with the MAP-2 of mammalian brain microtubules. One point which should be emphasized here is that of the total HMW fraction of brain microtubules, only MAP-2 survives the heat treatment (see Fig. 3, lane 3'). Therefore the amplification provided by

examining supernates from heating only applies to MAP-2 and the results presented here allow no conclusion to be drawn concerning the presence or absence in HeLa extracts of MAP-1 or other proteins in this molecular weight range.

The second antibody tested was a monoclonal antibody prepared against hog brain MAP-2 (21). The specificity of reaction of this antibody with calf brain microtubules and purified calf brain MAP-2 is shown in Fig. 5 (lanes 1–4). Again, there is also some reaction with lower molecular weight species, presumably proteolytic fragments. This antibody also reacts specifically with the protein present in the supernate prepared from heated two-cycle HeLa microtubules which comigrates with MAP-2 (lanes 5–6). In agreement with preliminary results reported by others (32), the antibody against MAP-2 does not react with the HeLa 201,000–206,000 mol wt MAPs. As shown above, the anti-HMW antibody does not react with the HeLa 201,000–206,000 mol wt MAPs either. These results suggest that the HeLa 201,000–206,000 mol wt proteins are not directly related to either MAP-1 or MAP-2 of brain.

Using both of the antibodies just discussed, preliminary immunofluorescence microscopy has been carried out in an attempt to detect the low levels of MAP-2 within HeLa cells and to study the intracellular distribution of MAP-2. Using the anti-HMW antibody, a fibrous intracellular network can be detected (Fig. 6a). This network disappears after colcemid treatment (Fig. 6d), and staining of the fibers can be blocked by pretreatment of the antibody with MAP-2 prepared by boiling pig brain microtubules (Fig. 6c). This network has not

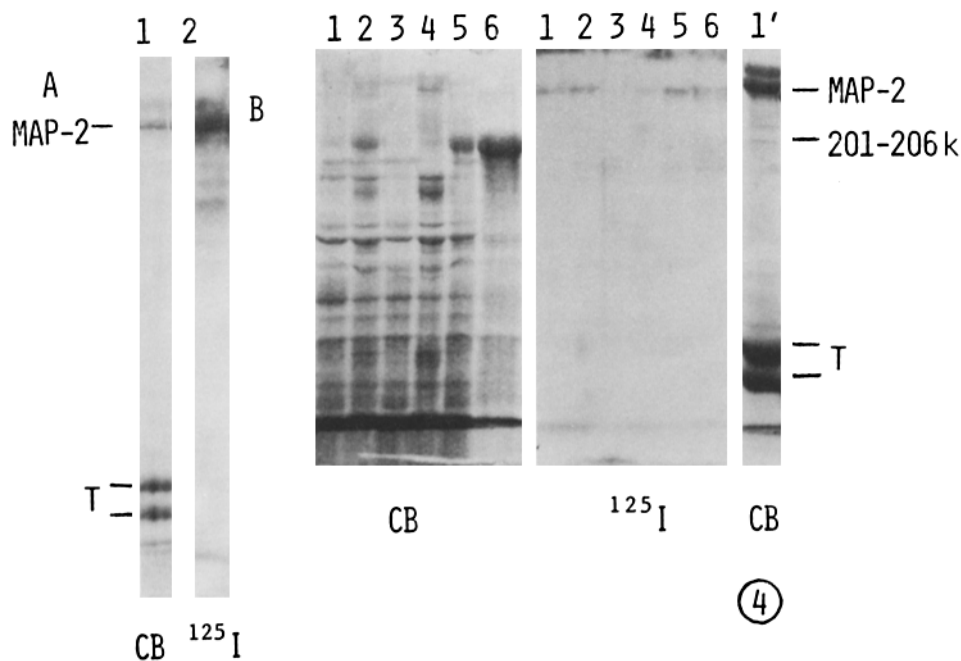


FIGURE 4 Interaction of anti-HMW antibody with heat-treated supernates from samples taken during a HeLa microtubule purification. (A, lane 1) Two-cycle brain microtubules (2.5 μ g) stained with Coomassie Blue (CB). (Lane 2) Autoradiograph of a gel identical to that in lane 1 after treatment with an anti-HMW (rat brain) antibody (36, 37) detected by treatment with 125 I-Protein A (see text for details). (B) At left and far right are gels stained with Coomassie Blue (CB). Lanes 1-6 represent heated supernates taken during the course of preparation of HeLa microtubules by polymerization-depolymerization cycles (corresponding to lanes 2-7 of Fig. 3). (Lane 1) 35,000 g_{max} supernate of sonicated cells. (Lane 2) First polymerization pellet. (Lane 3) First polymerization supernate. (Lane 4) First depolymerization pellet. (Lane 5) First depolymerization supernate. (Lane 6) Second depolymerization supernate (two-cycle microtubules). (Lane 1') Two-cycle calf brain microtubules. Each lane contains 30 μ g of protein. T indicates tubulin bands. The middle group of samples (designated 125 I) is an autoradiograph of an unstained gel identical to that on the left but treated with the anti-rat brain HMW antibody used in A, detected by binding of 125 I-Protein A.

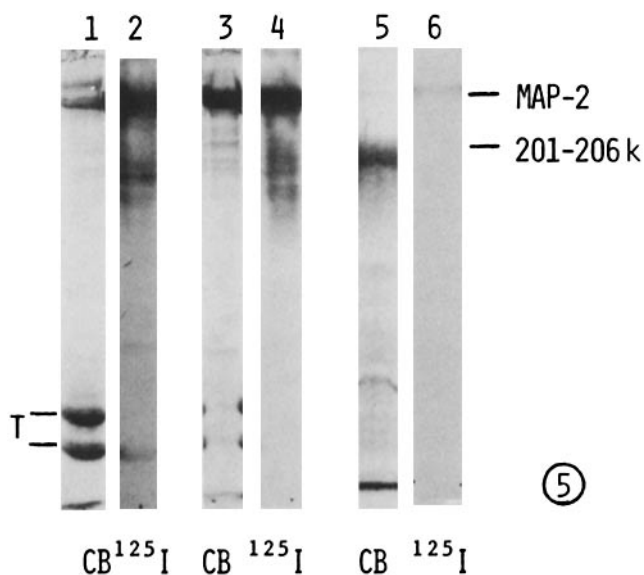


FIGURE 5 Reaction of a monoclonal antibody prepared against MAP-2 from hog brain microtubules with the heated supernate of two-cycle HeLa microtubules. The left gel in each pair is a stained gel (CB) and the right gel in each pair (125 I) is an autoradiograph of an identical gel following treatment with anti-MAP-2 and 125 I-Protein A (see text for details). (Lanes 1 and 2) Two-cycle calf brain microtubules (10 μ g). Lanes (3 and 4) MAP-2 purified from two-cycle calf brain microtubules (3 μ g). (Lanes 5 and 6) Supernate from centrifugation of heated two-cycle HeLa microtubules (30 μ g). T indicates tubulin bands.

been visualized to date in HeLa cells using the monoclonal antibody (21; B. D. Miles and J. A. Weatherbee, unpublished observations). Staining with the monoclonal antibody may be less sensitive since this antibody reacts with only a single determinant on the MAP-2 molecule, whereas the other antibody may react with several sites on each MAP-2 molecule. Additionally, there may be some contribution to the staining from MAP-1 or other proteins in this molecular weight range when the anti-HMW antibody is used. The observation that staining can be blocked by preadsorption with boiled MAP-2 suggests, however, that the bulk of the fibrous staining is due to MAP-2.

DISCUSSION

The 200,000 mol wt group of HeLa cell MAPs remains soluble after boiling microtubules or cell extracts, and the 200,000 mol wt MAPs, isolated by gel filtration of the heat soluble fraction of two-cycle microtubules, retain the ability to promote polymerization of tubulin. In these properties, therefore, the 200,000 mol wt MAPs resemble MAP-2 and tau. However, the 200,000 mol wt MAPs do differ from MAP-2 and tau in other respects, such as subunit molecular weight, reactivity with antibodies, and the ability of native subunits to self-associate (6). The 200,000 mol wt MAPs therefore appear to be a distinct class of quantitatively associated MAPs which share certain properties with MAP-2 and tau.

In addition, the following criteria indicate that HeLa cells contain a protein which almost certainly is MAP-2. This protein (a) comigrates with mammalian brain MAP-2, (b) is present in

cycled HeLa microtubules, (c) is heat stable, and (d) reacts with antibodies prepared against mammalian brain MAP-2. The antibody staining of the HeLa protein suggests, additionally, that this protein migrates as a closely spaced doublet on SDS PAGE, as does MAP-2 from brain (Fig. 4B, lane 6). Although we are not in a position to resolve all of the discrepant claims for the presence of MAP-2 in nonneural cells (see Introduction), our results and those of Cleveland et al. (10) indicate that MAP-2 is present in low levels in at least some nonneural cell lines. The heating procedure described in this report should provide an easy, direct way to screen for this protein in nonneural cells and should allow quantitative comparisons of the levels of MAP-2 present in various cell lines.

Previous studies that reported the absence of HMW-MAPs from cycled HeLa microtubules (3, 44, 45) are subject to the criticism that HMW-MAPs might be present in considerable quantities in cells, but are lost during cycling because of proteolysis. If proteolysis of HMW-MAPs were a problem, then one might argue that the 200,000 mol wt proteins which cycle with the microtubules are fragments of HMW proteins. The currently available evidence makes this unlikely. In the first place, the heating procedure permits the detection of MAP-2 in crude extracts before they have been subjected to

protracted incubations at 37°C and presumably before much proteolysis can have occurred. In fact the results actually indicate that although MAP-2 is present in cells and is partially lost during cycling, at no time is MAP-2 more than a minor component in comparison to the 200,000 mol wt MAPs. Densitometry of gels at the earliest stage of microtubule purification where both groups of proteins can be detected (the 35,000 g_{max} supernate of sonicated cells) shows that the ratio of the 200,000 mol wt MAPs to MAP-2 is in the range of six to nine on a weight basis. The second indication that the 200,000 mol wt MAPs are not fragments of HMW-MAPs comes from the observations that none of the available antibodies against either of these types of MAPs reacts with the other type of MAPs (4, 32; results presented here).

The reason for the loss of a large portion of the MAP-2 in the pellet of the first depolymerization step is not known. Tubulin is apparently a very minor component of this fraction (Fig. 3, untreated samples, lane 5) and yet two-thirds of the MAP-2 is lost in this fraction. One possible explanation is the suggestion of Pytela and Wiche (33), i.e., that HMW-MAPs may be lost by association with intermediate filaments. In the purification procedure followed here, using a fairly low-speed spin after cell disruption, the first depolymerization pellet is

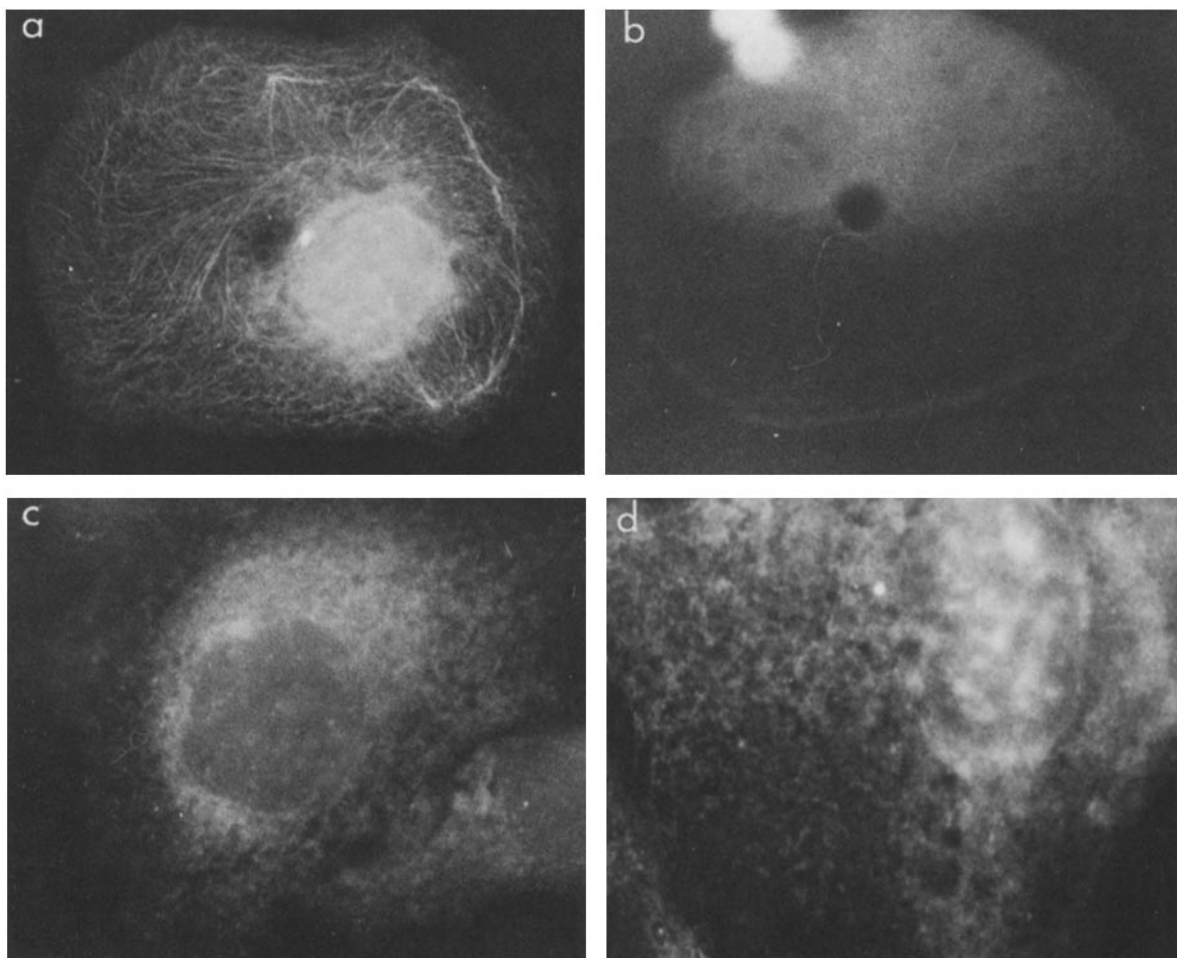


FIGURE 6 Indirect immunofluorescence microscopy of interphase HeLa cells using anti-H antibody. HeLa cells grown on coverslips were fixed and treated with an antibody prepared in rabbit against the total HMW-MAP fraction of rat brain microtubules. The antibody was detected by treatment with fluorescein-labeled goat anti-rabbit IgG antibody (see text for details). (a) Reaction of the antibody with an interphase HeLa cell. $\times 400$. (b) Control using pre-immune rabbit serum. $\times 400$. (c) Staining of interphase HeLa cells by the antiserum following preadsorption of the antiserum with MAP-2 prepared from boiled pig brain microtubules. $\times 400$. (d) Reaction of the antiserum with HeLa cells which had been exposed to 10^{-6} M Colcemid for 2 h before staining. $\times 1,000$.

the fraction in which intermediate filaments should be found. This fraction does contain a major protein band with a molecular weight of 53,000 (Fig. 3, heat-treated supernates, lane 5, in the region of the upper tubulin band) which could represent vimentin or one of the prekeratin subunits of intermediate filaments (25, 31). To this extent, these results are consistent with such a suggestion. This suggestion remains highly speculative, however, requiring identification of intermediate filaments in this fraction and demonstration of their interaction with MAP-2. If this suggestion is true, it could explain why microtubules from some cell types can not be repeatedly polymerized and depolymerized *in vitro* (see, e.g., Solomon et al. [41]) and would suggest that MAP-2 can bind to both microtubules and intermediate filaments and might mediate the interaction between these elements.

It is not immediately obvious what the property of high thermal stability indicates. Presumably it reflects a high degree of strong intramolecular bonding and is an indication of great structural stability. It seems quite unusual that this property is shown by several of the best-characterized MAPs; tau (8, 9), MAP-2 (23), and, as reported here, the HeLa 200,000 mol wt MAPs. It is particularly unusual for the latter two types of proteins in view of their high subunit molecular weights. Whatever the significance of heat stability, this property certainly simplifies purification of the 200,000 mol wt HeLa MAPs and MAP-2 and allows the detection of these proteins even in crude cell extracts.

Finally, it is not yet clear what function MAP-2 has in HeLa cells. Its concentration is low even in comparison to the 200,000 mol wt MAPs which are themselves relatively minor cellular components (estimated to account for about 0.04% of the total soluble cellular protein [4]). It is not surprising, therefore, that previous studies have failed to detect it (assuming that the differences between previous studies and the present one are not due to differences in strains of HeLa cells or growth conditions). This low level of MAP-2 might be attributable to gene leakage or to a peculiarity of an abnormal cell line grown in culture. On the other hand, MAP-2 might be serving a very specific function or might be associated only with certain classes of microtubules or might be expressed at different levels under different conditions. The immunofluorescence microscope observations with the anti-HMW antibody indicate that the MAP-2 in HeLa cells is associated with microtubules. It should become clearer whether or not MAP-2 has a specific function in nonneural cells when a variety of cell types besides HeLa have been examined for the presence of MAP-2 and the relative amounts of MAP-2 and any 200,000 mol wt MAPs have been determined.

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