Purification and Reconstitution of HeLa Cell Microtubules†

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ABSTRACT: Microtubules from suspension cultures of HeLa cells have been purified by carrying them through four complete cycles of polymerization at 37 °C and depolymerization at 4 °C. These microtubules show, in addition to the major α- and β-tubulin components, major proteins with molecular weights of 201 000–206 000 (comprising 4.5% of the total protein), proteins with molecular weights of 97 000, 100 000, 104 000, and 114 000 (together comprising ~2% of the total protein), and minor components with molecular weights of 68 000 and 151 000. HeLa microtubules have also been reconstituted from purified HeLa tubulin and proteins from HeLa microtubules separated from tubulin by DEAE-cellulose column chromatography. Experiments on the fractionation and reconstitution of both two- and four-cycle microtubules suggest that the 201 000–206 000-dalton proteins are incorporated into microtubules and promote tubulin polymerization. Microtubules formed by fractionation and reconstitution of two-cycle microtubules also contain several other proteins with molecular weights of 132 000, 146 000, 151 000, 160 000, and 284 000, although these are not present in microtubules carried through four assembly–disassembly cycles. Evidence is also presented which shows that a 68 000-dalton protein which is a prominent component of HeLa microtubules after two polymerization–depolymerization cycles does not stoichiometrically copurify with tubulin through repeated assembly–disassembly cycles and does not stimulate tubulin polymerization. On the other hand, the sedimentation of this 68 000-dalton protein is apparently influenced by the presence of polymerized microtubules, suggesting that this protein may be a component of a system which interacts weakly with microtubules. Finally, evidence is presented suggesting that two-cycle microtubules contain a proteolytic activity that can digest the 201 000–206 000-dalton proteins.

The study of the factors which control microtubule polymerization in vitro and possibly in vivo has only recently been extended to cultured cell systems. Reports have now appeared of the in vitro polymerization of microtubules from C_6 glial cells (Nagle et al., 1977; Wiche & Cole, 1976), normal and transformed 3T3 cells (Nagle et al., 1977; Weber et al., 1977; Wiche et al., 1977), Chinese hamster ovary cells, and neuroblastoma cells (Nagle et al., 1977). Other investigators have reported the association of proteins from cultured cell extracts with tubulin from mammalian brain microtubules (Cleveland et al., 1979; Klein et al., 1978; Seeds & Maccioni, 1978; Solomon et al., 1979). These studies have been limited by the small amounts of material available due to the reported necessity for the use of substrate-grown cells, by the need to copolymerize with mammalian brain microtubule proteins as carrier, and by the practice of using glycerol to promote tubulin polymerization rather than being able to rely on the polymerization-promoting activity of factors present in cell extracts. We have recently reported a procedure for the in vitro polymerization of microtubules from suspension-grown cultures of HeLa cells (Weatherbee et al., 1978). The use of suspension cultures allowed us to grow and harvest relatively large numbers of cells conveniently, and the use of glycerol to promote polymerization of tubulin was found not to be necessary. We found that the composition of HeLa microtubules purified through two cycles of polymerization and depolymerization as monitored by $NaDodSO_4$[1]–polyacrylamide gel electrophoresis was quite different from similarly cycled brain microtubules in terms of the nontubulin proteins present. No proteins were present which corresponded in size to either the HMW/MAP (high molecular weight/microtubule-associated proteins) (Dentler et al., 1975; Murphy & Borisy, 1975) or τ proteins (Weingarten et al., 1975). Instead, our preparations contained a number of other nontubulin proteins, the one present in highest concentration having a molecular weight of ~68 000. Shortly after submission of our results for publication, a report appeared which described the isolation of a protein from calf brain with a similar molecular weight (TAP, tubulin assembly protein) which promoted assembly of tubulin and which was incorporated into microtubules (Lockwood, 1978). It was also claimed (data not shown) that a similar TAP had been isolated from WI-38 and HeLa cells (Lockwood, 1978). A very recent report (Solomon et al., 1979) has also implicated a protein of about the same molecular weight

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[1] Abbreviations used: HMW/MAP, high molecular weight microtubule-associated proteins; TAP, tubulin assembly protein; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); GTP, guanosine 5′-triphosphate; DTE, dithioerythritol; PMSF, phenylmethanesulfonyl fluoride; TAME, *p*-tosyl-L-arginine methyl ester hydrochloride; DEAE, diethylaminoethyl; $NaDodSO_4$, sodium dodecyl sulfate; NQ-MAP, nonquantitatively purifying microtubule-associated protein; EDTA, ethylenediaminetetraacetic acid.

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as being associated with the microtubules of a hamster fibroblast cell line. Reports such as these as well as preliminary results of our own [presented in Weatherbee et al. (1978)] suggested that the 68 000-dalton protein found in our preparations might be a functional microtubule assessory protein and encouraged us to investigate further the properties of this protein.

To investigate whether the 68 000-dalton protein or other proteins present in our preparations could stimulate tubulin polymerization in vitro, we have extended our earlier studies as follows: (1) HeLa microtubules have been carried through an additional two cycles of polymerization and depolymerization to see which components present after the first two cycles continue to copurify with tubulin and promote its polymerization and (2) microtubules have been reconstituted from purified tubulin and certain protein fractions separated from microtubules by using DEAE-cellulose column chromatography. The results of these studies show that the 68 000-dalton protein does not stimulate tubulin polymerization and does not copurify stoichiometrically with tubulin through repeated assembly–disassembly cycles, although there is some evidence for an interaction of this protein with microtubules.

The results do suggest, however, other more likely candidates for microtubule-associated proteins in HeLa cells. The results of the repeated assembly–disassembly cycles show that several proteins do copurify with tubulin under these conditions. For convenience, we have grouped these proteins into two classes based on their molecular weights and their behavior on DEAE-cellulose columns. The first consists of at least two proteins in the molecular weight range of 201 000–206 000. The second consists of four major proteins in the molecular weight range of 97 000–114 000. Reconstitution experiments with the proteins of the 201 000–206 000-dalton class indicate that these proteins are capable of stimulating tubulin polymerization. Sufficient amounts of purified proteins of the second class have still not yet been obtained for conclusive testing of their ability to promote polymerization of tubulin.

During the course of this work, a report appeared (Bulinski & Borisy, 1979) in which microtubules from a different strain of HeLa cells were prepared in vitro by using a method similar to that which we had described in our earlier report (Weatherbee et al., 1978). These workers described the purification of HeLa microtubules through four assembly–disassembly cycles. The most prominent nontubulin proteins present had molecular weights of 210 000 and 120 000. Results were also presented on the fractionation of HeLa microtubules on DEAE-Sephadex A-50 columns. These showed that microtubules could be reconstituted from tubulin and the total MAP fraction, but the stimulation of tubulin polymerization by individual proteins of this fraction was not carried out. Although the correspondence in molecular weights is not exact, these results are clearly in general agreement with our observations. One discrepancy which remains, however, is that these workers do not find any 68 000-dalton protein present in major amounts in their preparations even in the earlier stages.

Materials and Methods

Cell Growth and Microtubule Preparation. HeLa cells (strain S-3) were grown in spinner bottles as described previously (Gruenstein et al., 1975). Microtubules were prepared from HeLa cells essentially as described previously (Weatherbee et al., 1978) with the modifications noted below. Our standard extraction buffer (PM) now contains 1 mM $MgSO_4$, 2 mM EDTA, 2 mM DTE, 100 KIU/mL Trasylol (aprotinin or kallikrein inactivator) (FBA Pharmaceuticals, New York), and 100 mM Pipes, pH 6.9. During extraction and depolymerization steps, this buffer contains 0.1 mM GTP (Type II-S, Sigma Chemical Co.). For microtubule polymerization, this concentration is increased to 2 mM. Cells were harvested, washed, and sonicated as described previously (Weatherbee et al., 1978). Following sonication, the high viscosity of the extracts was reduced by incubation with 0.1 mg/mL DNase I (Sigma Chemical Co.) for 15 min at 0 °C. Following this, extracts were centrifuged at $35000g_{max}$ (17 000 rpm in a Sorvall SS-34 rotor) for 30 min at 4 °C. Microtubules were then polymerized and collected from the supernate of this step by addition of GTP, incubation at 37 °C, and centrifugation as described previously (Weatherbee et al., 1978). Depolymerization was also as described previously, except that the centrifugation speed following depolymerization was increased to $100000g_{max}$ (33 000 rpm in a Spinco 50Ti rotor). In extending our earlier procedure through an additional two cycles of assembly–disassembly, the following suspension volumes were used: the first polymerization pellet was suspended in a volume of buffer equal to 1/5 of the volume of the initial $35000g$ supernate; the second polymerization pellet was suspended in a volume of buffer equal to 1/3 of the volume of the first depolymerization supernate; the third polymerization pellet was suspended in a volume of buffer equal to 1/2 of the volume of the second depolymerization supernate; the fourth polymerization pellet was suspended in 1/2 of the volume of the third depolymerization supernate. To obtain sufficient starting material to allow four polymerization–depolymerization cycles to be carried out, we used 6–8 L of medium with cell densities of 5–7 × 10^5 cells/mL. In some cases, microtubules were carried through all four assembly–disassembly cycles without interruption. In other cases, samples of the second depolymerization supernates were rapidly frozen in dry ice, stored overnight at −80 °C, thawed rapidly, and then carried through the two additional assembly–disassembly cycles. No major differences were found in the yield or composition of the microtubules prepared by either procedure.

A fairly high degree of variability in the amount of the 201 000–206 000-dalton doublet present in preparations after two cycles of assembly–disassembly was seen in early experiments. The inclusion of Trasylol seems to decrease this variability. However, as discussed under Results, preparations at this stage of purification still contain apparent proteolytic activity which can result in drastic reduction in the level of the 201 000–206 000-dalton proteins present following lengthy incubations during sample concentrations, even in the presence of Trasylol. Because of a general concern for the possible loss of components, e.g., proteins corresponding to the HMW/MAP proteins of neural microtubules, we have tested the effect of several protease inhibitors in our preparations. In addition to Trasylol, these have included leupeptin (50 μg/mL) and a combination of PMSF (0.1 mM) and TAME (1 mg/mL). Trasylol, as noted above, seemed to decrease the variability in the level of the 201 000–206 000-dalton proteins present. None of the protease inhibitors had any obvious effect in drastically increasing the level of the 201 000–206 000-dalton proteins present in two cycle preparations or in causing the appearance of any new higher molecular weight proteins in these preparations. PMSF and TAME were obtained from Sigma. Leupeptin was the gracious gift of Dr. R. C. Lucas, Downstate Medical Center, Brooklyn, NY.

Column Fractionation of HeLa Microtubules. Microtubules carried through two or four polymerization–depolymerization cycles were stored at −80 °C and material from

several preparations was pooled before column fractionation. The general conditions for fractionation of HeLa microtubules on DEAE-cellulose (Whatman DE-52, H. Reeve Angel & Co., Inc., Clifton, NJ) or phosphocellulose (Whatman P-11, H. Reeve Angel & Co., Inc.) were essentially as described previously (Weatherbee et al., 1978) with the exception that 2 mM DTE was included in all equilibration and elution buffers. Column fractions were pooled and concentrated by using CS-15 Minicon macrosolute concentrators (Amicon Corp., Lexington, MA). Column fractions eluted by NaCl were desalted on columns of Sephadex G-25 (medium) (Pharmacia Fine Chemicals, Inc., Piscataway, NJ). Alternatively, column fractions were simultaneously concentrated and dialyzed against PM buffer by using pressure dialysis. Pooled, concentrated, and desalted tubulin fractions were always centrifuged at $56600g_{max}$ for 30 min at 4 °C immediately prior to reconstitution experiments to remove aggregated protein. Protein concentrations were routinely determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Purified HeLa tubulin for reconstitution experiments was prepared by either of two methods. Small amounts of very pure tubulin could be prepared from two cycle HeLa microtubule preparations by adsorption to DEAE-cellulose and elution with a continuous 0–0.5 M NaCl gradient as described previously (Weatherbee et al., 1978) (designated "DE-gradient tubulin"). Slightly less pure tubulin can be prepared more rapidly and in higher yield by a somewhat different method. In this procedure, two-cycle HeLa microtubules were applied to a column of phosphocellulose equilibrated in PM buffer (column volume approximately equal to 1 mL/10 mg of protein). Fractions were monitored by OD_{280}, and the fractions of the unadsorbed peak of protein were pooled. NaCl was added to the pooled fractions to a concentration of 0.1 M, and the sample was applied to a DEAE-cellulose column equilibrated in 0.1 M NaCl in PM buffer (column volume approximately equal to 1 mL/4 mg of protein). The column was washed with an additional column volume of the same buffer, and tubulin was then eluted with 0.5 M NaCl in PM buffer. Tubulin prepared in this way (designated "PC/DE tubulin") contained only trace amounts of nontubulin proteins (see Figure 6a, lane 3). Tubulin prepared by either method showed no ability to form microtubules when incubated at concentrations of 1 mg/mL at 37 °C in 2 mM GTP in PM buffer, as monitored by negative-staining electron microscopy.

Preparation of Calf Brain MAP-2. Microtubules from calf brain were purified by two cycles of assembly–disassembly in the absence of glycerol. Fresh calf brains were homogenized in PM buffer (less DTE and Trasylol) (1.5 mL of buffer/g wet weight) by using a Sorvall Omnimixer. The homogenates were centrifuged for 30 min at 4 °C at 10000 rpm in a Sorvall GSA rotor. The GTP concentration of the supernate was adjusted to 1 mM, and microtubules were purified by polymerization and depolymerization as described above for HeLa microtubules. MAP-2 was prepared from two-cycle microtubules by the heat-treatment method of Fellous et al. (1977) as modified by Kim et al. (1979).

Standard Reconstitution Assay. Since HeLa tubulin prepared by either of the two procedures previously described did not form microtubules when incubated at a concentration of 1 mg/mL under conditions known to promote microtubule assembly, it was possible to use this tubulin to test the ability of subfractions of microtubules to promote formation of microtubules. Varying concentrations of nontubulin protein fractions were added to HeLa tubulin maintained at a concentration of 1 mg/mL at 0 °C in PM buffer. GTP was added to a final concentration of 2 mM and samples were incubated at 37 °C for 30 min. Samples were negatively stained for electron microscopy (EM) by using the protocol described previously (Weatherbee et al., 1978), and grids were examined for the presence or absence of microtubules in a Philips EM 301 electron microscope. When microtubules were found and sufficient material was available, the composition of the microtubules formed was determined by pelleting the microtubules at $56600g_{max}$ for 30 min at 35 °C and analyzing the pellet and supernate fractions on $NaDodSO_4$–polyacrylamide gel electrophoresis.

$NaDodSO_4$ Gel Electrophoresis. $NaDodSO_4$–polyacrylamide gel electrophoresis was carried out according to the method of Neville (1971) by using running gels of either 5 or 8% polyacrylamide as indicated. The staining and destaining protocol of Fairbanks et al. (1971) was used. Densitometry of gels was done by using a 570-nm filter in a Helena Quick Scan, Jr. (Helena Laboratories Corp., Beaumont, TX), modified from the standard apparatus so as to have smaller slits and a linear optical bench.

The following proteins were used as electrophoretic standards: rabbit myosin (Kielley & Bradley, 1956), pig α-actinin (generously provided by Dr. Darrel Goll), rabbit actin (Spudich & Watt, 1971), bovine serum albumin, and α-chymotrypsinogen (Sigma Chemical Co., St. Louis, MO).

Results

Composition of HeLa Microtubules Carried through Four Cycles of Polymerization and Depolymerization. Because of the large number of nontubulin components present after two cycles of polymerization and depolymerization, some of which probably represent contaminants with no role in microtubule function, it was important to try to purify HeLa microtubules more extensively than in our earlier work (Weatherbee et al., 1978) by starting with larger amounts of material so we could carry out additional cycles of polymerization and depolymerization. This enabled us to determine which of the nontubulin components present after two cycles would continue to cycle with tubulin.

Figure 1 shows the $NaDodSO_4$ gel electrophoresis analysis of a HeLa microtubule preparation which had been carried through four assembly–disassembly cycles. In the supernate of the second depolymerization, the 68000-dalton protein was, as reported previously, the major nontubulin protein present (lane 10). However, this 68000-dalton protein was clearly diminished in the third polymerization pellet (lane 11) and enriched relative to tubulin in the supernate from this polymerization (lane 12). This pattern of loss of the 68000-dalton protein was repeated at the fourth polymerization step (lanes 15 and 16). By the end of four complete cycles of polymerization and depolymerization, the 68000-dalton protein was greatly decreased (lane 18). Instead, $NaDodSO_4$–polyacrylamide gel electrophoresis analysis of preparations at this stage of purification showed, in addition to the major tubulin bands, a prominent band at ~206000 daltons. At lower concentrations, this band can be resolved into two closely spaced bands with molecular weights of about 201000 and 206000. Also reproducibly present in four cycle HeLa microtubule preparations are a triplet of bands at 97000, 100000, and 104000 daltons (see also Figure 5, lane 1), a band at 114000 daltons, the 68000-dalton band, and a very faint band at 151000 daltons. In contrast to the 68000-dalton protein, it can be seen that the 201000–206000- and 97000–114000-dalton proteins are completely included in the microtubules pelleted at the third and fourth polymerization steps

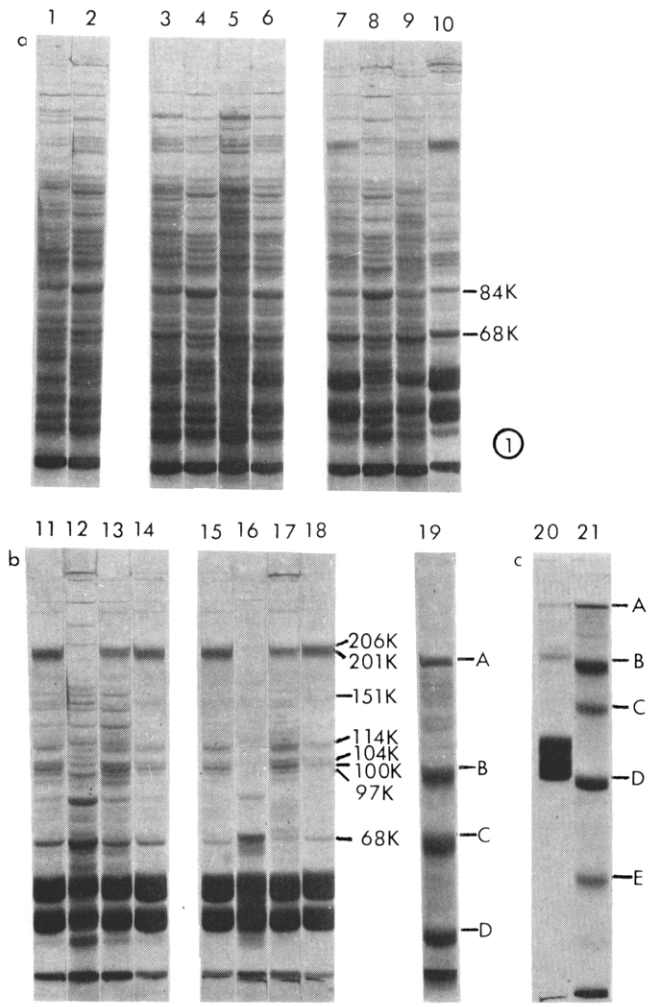

FIGURE 1: Purification of HeLa microtubules through four polymerization–depolymerization cycles. (a) First cycle: (1) $35000g_{max}$ pellet; (2) $35000g_{max}$ supernate; (3) polymerization pellet; (4) polymerization supernate; (5) depolymerization pellet; (6) depolymerization supernate. Second cycle: (7) polymerization pellet; (8) polymerization supernate; (9) depolymerization pellet; (10) depolymerization supernate. (b) Third cycle: (11) polymerization pellet; (12) polymerization supernate; (13) depolymerization pellet; (14) depolymerization supernate. Fourth cycle: (15) polymerization pellet; (16) polymerization supernate; (17) depolymerization pellet; (18) depolymerization supernate. (19) Molecular weight standards: A, myosin heavy chain (200 000); B, α-actinin (100 000); C, bovine serum albumin (69 000); D, actin (42 000). 50 μg of protein was applied to each gel (1–18). Gels were 5% polyacrylamide. (c) (20) Fourth cycle depolymerization supernate (50 μg of protein) run on 8% polyacrylamide to demonstrate absence of any proteins with molecular weights less than actin; (21) molecular weight standards on 8% polyacrylamide: A–D as above; E, α-chymotrypsinogen (25 700).

(lanes 11 and 15) and completely removed from the supernates at these steps (lanes 12 and 16). It can also be seen, however, that small amounts of these proteins, as well as tubulin, are being lost in the pellets at the third and fourth depolymerization steps (lanes 13 and 17), apparently as a result of incomplete depolymerization at these steps. Figure 1 also shows a sample of the fourth depolymerization supernate run on an 8% polyacrylamide gel (lane 20), showing that there are no major proteins in the molecular weight region below actin, an area not resolved on the standard 5% gels.

Taken together, the proteins in the range of 97 000–114 000 daltons account for ~2% of the total protein by the end of the third cycle and are maintained at this level at the end of the fourth cycle. The 201 000–206 000-dalton proteins account for ~4.5% of the total protein through the third and fourth

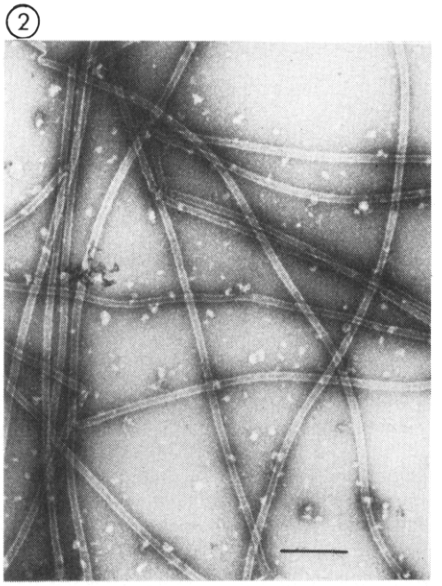

FIGURE 2: HeLa microtubules formed during a fourth polymerization cycle. 36 000× magnification. Bar = 0.25 μm.

cycles. The 68 000-dalton protein, which accounted for ~5% of the total protein after two assembly–disassembly cycles, decreases to ~1% of the total protein after three cycles and to less than 1% after four cycles. Tubulin accounts for ~81% of the total protein following two cycles, 89% of the total following three cycles, and 90–92% following four cycles. A negatively stained sample of the microtubules formed at the fourth polymerization step is shown in Figure 2.

It should be pointed out that there are considerable losses of material in the third and fourth polymerization cycles. Of the total protein present after two cycles, only 26–36% (range for four preparations) of this protein is carried through an additional polymerization–depolymerization cycle, and only 6–11% is carried through a fourth cycle. This is despite the fact that microtubules certainly accounted for the majority of the protein present following the second assembly–disassembly cycle (tubulin alone accounts for 81% of the protein at this stage). These losses could result either from a gradual generalized loss of the ability to polymerize reversibly during the course of purification, owing to a gradual denaturation of tubulin or of associated proteins which are facilitating polymerization and depolymerization, or from the presence of different classes of microtubules with differing stabilities. These considerations suggest that the use of repeated in vitro assembly–disassembly cycles may select for only the most stable microtubule accessory proteins or the most stable classes of microtubules and not necessarily for all accessory proteins or microtubule classes which are present in vivo.

Partial Purification and Properties of the 68 000-Dalton Protein. Because of its prominence in twice-cycled HeLa microtubule preparations, we tested the ability of the 68 000-dalton protein to promote polymerization of tubulin. Since these experiments make it seem less likely that the 68 000-dalton protein is a microtubule accessory protein, the results of these studies will be only briefly summarized here.

The 68 000-dalton protein has been partially purified by taking advantage of the fact that in standard PM buffer this protein is largely not adsorbed to either phosphocellulose or DEAE-cellulose (Weatherbee et al., 1978). By application of a two-cycle microtubule preparation to phosphocellulose and then application of the unadsorbed fraction to DEAE-cellulose, a fraction can be obtained in which the most prominent band (well over half the bound dye) was the 68 000-dalton protein.

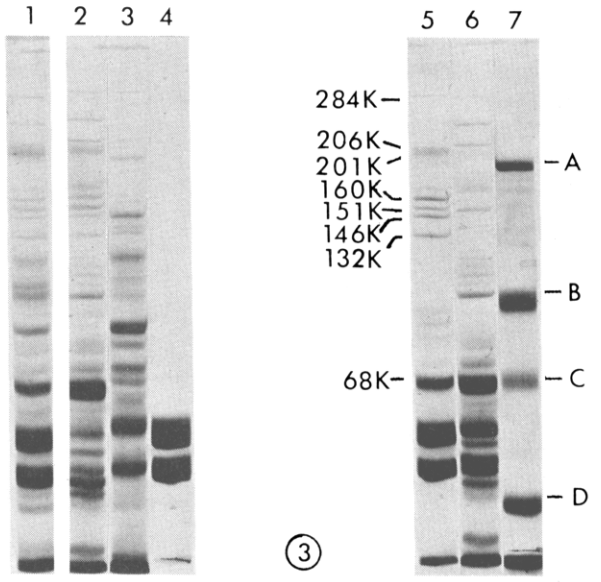

FIGURE 3: Composition of microtubules reconstituted from DEAE-cellulose nonadsorbed fractions and tubulin. (1) Unfractionated two-cycle HeLa microtubules. (2) Nonadsorbed fraction from DEAE-cellulose [corresponding to peak I of Figure 7 of Weatherbee et al. (1978)]. (3) Protein eluted in early part of the gradient [corresponding to peak II of Figure 7 of Weatherbee et al. (1978)]. (4) Tubulin eluted in later part of the gradient [corresponding to peak III of Figure 7 of Weatherbee et al. (1978)]. (5) Microtubules from the pellet of centrifugation of a combination of samples shown in gels 2 and 4 after incubation at 37 °C with 2 mM GTP. (6) Supernate from the pellet shown on gel 5. (7) Molecular weight standards A–D as indicated in Figure 1. 50 μg of protein was applied to each gel. All gels were 5% acrylamide.

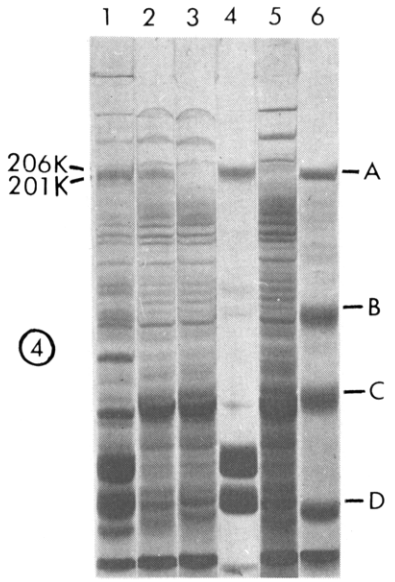

FIGURE 4: Loss of 201 000–206 000-dalton proteins during concentration of DEAE-cellulose unadsorbed fractions. (1) Two-cycle HeLa microtubules. (2) DEAE-cellulose unadsorbed fraction of two-cycle HeLa microtubules. (3) DEAE-cellulose unadsorbed fraction following simultaneous concentration and dialysis (threefold concentration; ~12 h at 4 °C). Note the apparently specific loss of 201 000–206 000-dalton proteins. (4) Four-cycle HeLa microtubules. (5) Same sample as lane 3, applied at a higher concentration to show that low levels of the 201 000–206 000-dalton proteins remain in this fraction. (6) Molecular weight standards A–D as indicated in Figure 1. Gels in lanes 1, 4, and 5 contained 50 μg of protein. Gels in lanes 2 and 3 contained 29 μg of protein. All gels were 5% polyacrylamide.

The most prominent contaminants had molecular weights of 47 000 and 53 000, and a few other minor components were also present. In a series of experiments, we added up to 0.2 mg/mL of this 68 000 dalton enriched fraction to DE-gradient tubulin, incubated the samples at 37 °C for 30 min with 2 mM GTP in PM buffer, and searched for microtubules as decribed under Materials and Methods. No microtubules were ever found. On the other hand, as discussed below, we have routinely been able to reconstitute HeLa microtubules by the addition of other microtubule fractions to purified HeLa tubulin, and we feel that this result is a reliable indication that the 68 000-dalton protein does not promote tubulin polymerization at the concentration we tested.

DEAE-cellulose Fractionation and Reconstitution Experiments. Next, we investigated the ability of other nontubulin components of HeLa microtubules to promote polymerization of pure HeLa tubulin at 1 mg/mL. We chose to investigate those nontubulin proteins of two- and four-cycle microtubules which are not adsorbed to DEAE-cellulose because these fractions contain the 201 000–206 000-dalton components present in four-cycle microtubules but do not contain the 97 000–114 000-dalton proteins. The latter observation is most clearly shown in the fractionation of four-cycle microtubules (Figure 5) but is also evident in the fractionation of two-cycle microtubules (Figure 3). We were therefore in a position to determine if a particular set of the proteins present in four-cycle HeLa microtubules was individually capable of supporting polymerization of purified HeLa tubulin.

Tests of the ability of these fractions to promote tubulin assembly required concentration of the DEAE-cellulose unadsorbed fractions. This led to the unexpected finding that the unadsorbed fraction of two-cycle HeLa microtubules contains an apparent proteolytic activity to which the 201 000–206 000-dalton proteins are extremely sensitive. This activity results in a drastic decrease in the amount of these proteins present in DEAE-cellulose unadsorbed fractions following concentration preparatory to recombination with tubulin (Figure 4). The 201 000–206 000-dalton proteins are the only components in these preparations which are obviously decreased during concentration, suggesting that these proteins are particularly sensitive to proteases or that there is some specificity in the protease activity. This observation may explain the variability we have seen in the amount of the 201 000–206 000-dalton proteins present in two-cycle preparations and suggests the possibility that cells may contain higher amounts of these proteins than are found in microtubules polymerized from crude extracts or even that the yield of microtubules which can be polymerized in vitro is reduced because of the loss of these proteins during isolation. Work is presently being carried out to identify the products formed by digestion of the 201 000–206 000-dalton proteins and to determine how to inhibit endogenous digestion. In contrast, the DEAE-cellulose unadsorbed fraction of four-cycle microtubules can be concentrated without appreciable loss of the 201 000–206 000-dalton proteins (Figures 5 and 6). This shows that the presumptive protease is removed by repeated assembly–disassembly cycles and rules out the possibility that the 201 000–206 000-dalton proteins are being lost during concentration by selective adsorption to dialysis tubing or the walls of the concentrator.

Although the concentration of the 201 000–206 000-dalton proteins in DEAE-cellulose unadsorbed fractions of two-cycle microtubules is greatly decreased upon concentration, these proteins do not completely disappear (Figure 4, lane 5), and several reconstitution experiments have been carried out in which this fraction has been added to tubulin and the reconstituted microtubules have been identified by electron microscopy and analyzed by $NaDodSO_4$–polyacrylamide gel

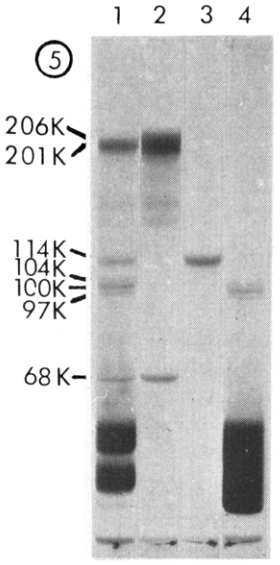

FIGURE 5: DEAE-cellulose fractionation of four-cycle HeLa microtubules. (1) Unfractionated four-cycle microtubules. (2) DEAE-cellulose unadsorbed fraction. (3) Fraction eluted by 0.1 M NaCl in PM buffer. (4) Fraction eluted by 0.5 M NaCl in PM buffer. A 60-μL sample of the peak fraction of each elution condition was applied to each gel. All gels were 5% polyacrylamide.

electrophoresis. In one such experiment (Figure 3), the concentration of unabsorbed protein (Figure 3, lane 2) in the incubation mixture was 2.23 mg/mL, and the tubulin (Figure 3, lane 4; DE-gradient tubulin) was at a concentration of 1 mg/mL in a total volume of 1.0 mL. This high concentration of unadsorbed protein in relation to that of tubulin was added because it was anticipated from the four-cycle results that only a few of the proteins present in this fraction would be incorporated into microtubules and because we were concerned that the reduced concentration of the 201 000–206 000-dalton components would be insufficient to promote tubulin polymerization unless crude fractions were added at high levels. Electron microscopy of the sample (not shown) following incubation with 2 mM GTP at 37 °C for 30 min showed the presence of numerous microtubules. The microtubules recovered by centrifugation at $56600g_{max}$ contained a total of 0.75 mg of protein. NaDodSO$_4$–polyacrylamide gel electrophoresis of this sample (Figure 3, lane 5) showed that tubulin accounted for the bulk of the protein, suggesting that nearly three-quarters of the tubulin was polymerized.

Additional inspection of the gel electrophoretic pattern of these microtubules showed a quite reproducible pattern of nontubulin proteins. As might have been predicted from the results of repeated temperature cycling, the reconstituted microtubules (Figure 3, lane 5) show the presence of the 201 000–206 000-dalton proteins. These proteins appear to be present only in the pelleted microtubules (Figure 3, lane 5) and to have been completely removed from the supernate (Figure 3, lane 6). As mentioned previously, the proteins in the range of 97 000–114 000 daltons which copurify with tubulin through four temperature cycles are not present in the DEAE-cellulose unadsorbed fractions of two-cycle microtubules and therefore would not be expected in these reconstituted microtubules.

Two unexpected differences between the results of the temperature-cycling experiments and these reconstitution experiments are apparent. The first is the presence in the reconstituted microtubules of four prominent bands with molecular weights of about 132 000, 146 000, 151 000, and 160 000 (Figure 3, lane 5). These bands are seen in unfractionated microtubules after two polymerization–depolymerization cycles (Figure 3, lane 1) but generally do not copurify through four cycles, although trace amounts of the 151 000-dalton protein can be seen in four-cycle preparations (Figure 5, lane 1). As was the case for the 201 000–206 000-dalton proteins, three of these bands are present only in the microtubule pellet and are completely removed from the supernate. The significance of these proteins is not at present clear. The possibility that any of these proteins is a product resulting from digestion of the 201 000–206 000-dalton proteins is presently being investigated. A few other minor components also appear to be incorporated into the reconstituted microtubules, in-

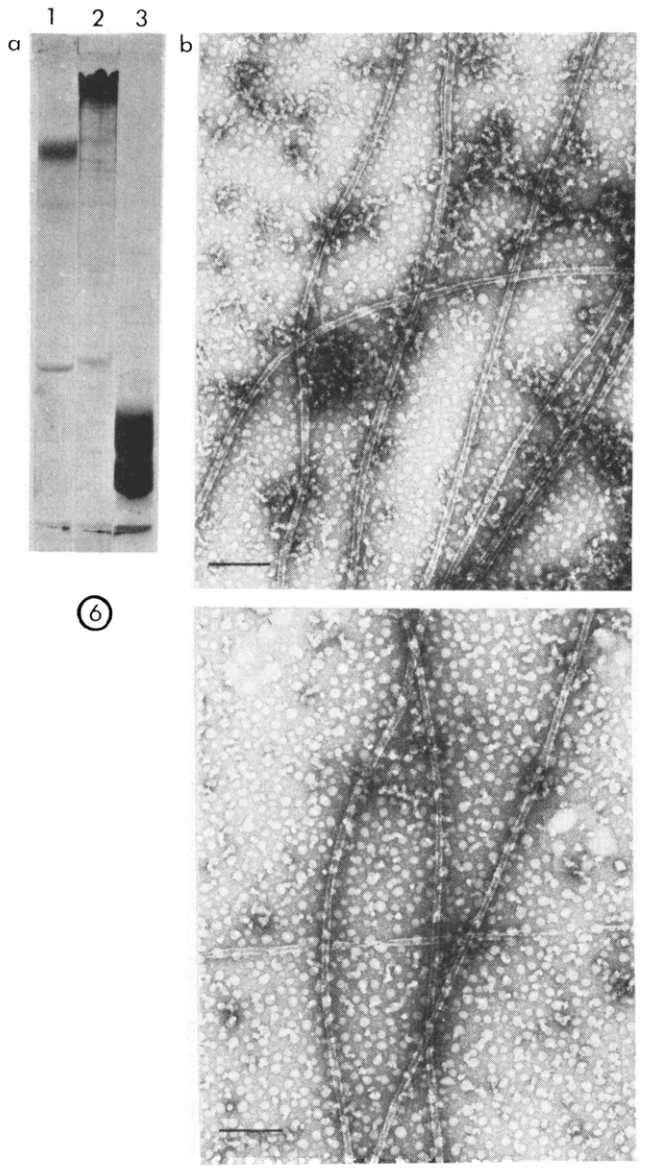

FIGURE 6: Promotion of tubulin polymerization by nontubulin fractions. (a) (1) 201 000–206 000 dalton protein enriched fraction [DEAE-cellulose unadsorbed fraction from four-cycle HeLa microtubules (see Figure 5, lane 2) after concentration and dialysis] (5 μg of protein); (2) MAP-2 prepared from two-cycle calf brain microtubules (10 μg of protein); (3) HeLa tubulin prepared by column chromatography on consecutive columns of phosphocellulose and DEAE-cellulose (see Materials and Methods) (50 μg of protein). (b) Microtubules polymerized by combining fraction shown in lane 1 (201 000–206 000-dalton proteins plus 68 000-dalton protein) (final concentration = 0.29 mg/mL) with HeLa tubulin preparation shown in lane 3 (final concentration = 1.0 mg/mL). 36 000× magnification. Bar = 0.25 μm. (c) Microtubules polymerized by combining fraction shown in lane 2 (MAP-2) (final concentration = 0.29 mg/mL) with HeLa tubulin preparation shown in lane 3 (final concentration = 1.0 mg/mL). 36 000× magnification. Bar = 0.25 μm.

cluding a high molecular weight component (~284 000 daltons) which is also seen in two-cycle but not four-cycle microtubules.

The second unexpected difference between the reconstitution results and the previously discussed results from four assembly–disassembly cycles is the presence of substantial amounts of the 68 000-dalton protein in the pellet of reconstituted microtubules in all experiments of this type. (The concentration of the 68 000-dalton protein in the pellet of reconstituted microtubules is more than 100-fold higher than the concentration of this protein present in a control pellet formed when the unadsorbed fraction was incubated in the absence of tubulin at the same concentration and under the same conditions as in the reconstitution experiment described above, as determined by scans of the respective gels.) The concentration of the 68 000-dalton protein in the microtubule pellet is also considerably higher than can be accounted for by simple contamination of the pellet by included supernate. These results clearly suggest an affinity of this protein for microtubules. On the other hand, it is clear that the bulk of the 68 000-dalton protein remains in the supernate (Figure 3, lane 6). Comparison of a number of reconstitution experiments showed that the ratio of the 68 000-dalton protein to tubulin was variable, suggesting that there is not a stoichiometric association of this protein with tubulin. These results, together with the previously discussed results from four temperature cycles and the failure of enriched preparations of the 68 000-dalton protein to promote tubulin polymerization, all suggest that this protein is not an integral microtubule protein.

Although the results from the fractionation of two-cycle microtubules are consistent with a role for the 201 000–206 000-dalton proteins in the promotion of tubulin polymerization, interpretation of the results is complicated by the large numbers of nontubulin proteins still present in preparations at this stage of purification and by the decreased levels of the 201 000–206 000-dalton proteins present in concentrated DEAE-cellulose unadsorbed fractions. Much cleaner results have been obtained from fractionation of four-cycle HeLa microtubules. Of course, studies of this type require much larger numbers of cells and entail smaller yields, so that only small-scale experiments have been possible so far. The result shown in Figure 5 was obtained by pooling the microtubules from four assembly–disassembly cycles from a total of 32 L of suspension cultures of HeLa cells (four batches). A total of ~7 mg of microtubule protein was obtained. These microtubules can be rapidly fractionated on DEAE-cellulose columns by application in standard buffer, followed by elution with two steps of 0.1 and 0.5 M NaCl in the same buffer. The unadsorbed fraction contains the 201 000–206 000-dalton proteins and the remnant of the 68 000-dalton protein (Figure 5, lane 2). The 114 000-dalton protein is eluted by 0.1 M NaCl (lane 3), and the 97 000–104 000-dalton proteins and tubulin are eluted by 0.5 M NaCl (lane 4).

We found that the fraction containing predominantly the 201 000–206 000-dalton proteins (>60% of the total protein) (Figure 5, lane 2, and Figure 6, lane l) can promote microtubule formation when added to purified HeLa tubulin in standard buffer containing 2 mM GTP as determined by examination of negatively stained EM grids of the preparations. The tubulin in these experiments was maintained as usual at 1.0 mg/mL ("PC/DE tubulin"; Figure 6a, lane 3). Occasional microtubules are seen on grids when concentrations of the 201 000–206 000-dalton plus 68 000-dalton protein fraction as low as 0.1 mg/mL are added, and numerous microtubules are seen when the concentration of this fraction is increased to 0.2–0.3 mg/mL (Figure 6). These levels are higher than the levels of these proteins in relation to tubulin found in unfractionated microtubules. However, a direct comparison of grids of samples containing tubulin plus equal concentrations of the HeLa proteins (Figure 6b) or calf brain MAP-2 (Figure 6c) shows comparable numbers of microtubules formed in either case. This indicates that the HeLa proteins are approximately as efficient in the promotion of tubulin polymerization as the brain MAP. The 68 000-dalton protein is a minor component of this DEAE-cellulose unadsorbed fraction (~8% of the total protein), and, therefore, its concentration in these experiments was much lower than in the experiments with partially purified 68 000-dalton protein which failed to show any effect of this protein on tubule polymerization. Thus, it seems clear that the 201 000–206 000-dalton proteins are the components of the DEAE-cellulose unadsorbed fraction of four-cycle microtubules which are responsible for promotion of tubulin polymerization in these experiments.

Discussion

Largely on the basis of work with neural microtubules, two general criteria have been advanced as the basis for classification of a nontubulin protein as a microtubule-associated protein. These are (1) copurification with tubulin at a constant stoichiometry through the course of repeated polymerization–depolymerization cycles in vitro and (2) the ability of a protein to promote the net assembly of microtubules under conditions which do not favor the polymerization of pure tubulin. Clearly, however, neither of these criteria is unequivocal. Circumstances can be envisioned under which a protein which was associated with microtubules in vivo would not copurify with tubulin in vitro. For example, a protein which served as a bridge between microtubules and actin or intermediate filaments could copurify with either microtubules or the other filament systems depending on its relative affinity for tubulin or actin or intermediate filament protein. Conversely, theoretical arguments have been presented which suggest that copurification with tubulin through repeated assembly–disassembly cycles does not necessarily prove a specificity of interaction with tubulin (Lee et al., 1978). As for the second criterion, there does not seem to be any overwhelming reason why all proteins which associate in vivo with microtubules should necessarily promote net microtubule formation in vitro. That the HMW/MAP and τ proteins of neural microtubule preparations show this property may be fortuitous, and, in any case, there is at present no evidence that these proteins perform such a function in vivo. Finally, it must be emphasized that these criteria are based on in vitro observations. Gradual denaturation of tubulin or associated proteins, loss of cytoplasmic cofactors and enzymes, and dephosphorylation of tubulin or associated proteins are only a few of the imaginable processes which may be occurring during in vitro manipulations of microtubules and which could affect the associations and activities of accessory proteins with respect to tubulin to produce results which do not reflect in vivo conditions.

With these qualifications in mind, however, the criteria mentioned above are valuable in preliminary studies for suggesting likely candidates for microtubule-associated proteins for further study, and these are the criteria which we have used in interpreting our experiments with HeLa microtubules. From all the results presented in this paper, the proteins with molecular weights of 201 000–206 000 have emerged as very strong candidates for microtubule-associated proteins in HeLa cells. These proteins appear to meet both of the criteria mentioned above, i.e., they copurify with tubulin through re-

peated assembly–disassembly cycles and promote tubulin polymerization in vitro. This is the first observation that a specific set of proteins, rather than a crude MAP fraction, can promote microtubule polymerization in this system. Other proteins with molecular weights in the range of 97 000–114 000 also represent likely candidates for further study. These proteins clearly copurify with tubulin. Insufficient amounts of purified preparations of these proteins have been available, however, to allow determination of whether these proteins affect tubulin polymerization. Other proteins are also possible candidates, but the evidence is less consistent.

On the other hand, by both of the criteria established above, the 68 000-dalton protein is not a microtubule-associated protein. Nevertheless, the reconstitution experiments and the consistent presence of low levels of this protein even in four-cycle preparations suggest an affinity of this protein for microtubules. On the basis of all of the observations presented, this protein presently must be categorized as an NQ-MAP according to the terminology suggested by Berkowitz et al. (1977). Whether this protein is related to other proteins of approximately the same molecular weight which other workers have implicated as being associated with the microtubules of other cultured cells (Solomon et al., 1979; Lockwood, 1978) remains to be determined.

In the experiments concerned with the effect of added components on tubulin polymerization, we have relied on the qualitative observation of the presence or absence or relative numbers of microtubules in negatively stained samples of preparations in which the tubulin concentration was maintained at $\sim$1 mg/mL. This type of assay was required by the much smaller amounts of protein available from HeLa microtubule preparations in comparison with brain microtubule preparations. Nevertheless, the results are clear-cut. Microtubules are never seen in pure tubulin samples incubated at this concentration, but numerous microtubules are found when appropriate fractions are added back to purified tubulin. In a sense, this assay is less ambiguous than others in that it monitors for the presence of normal microtubules. Other assays based on turbidity changes or sedimentation can be affected by the formation of aggregates or aberrant microtubules.

Despite the difficulties involved, the study of microtubules from HeLa cells is important to determine the similarities and differences between the proteins of these microtubules and those of neural microtubules since most current models for microtubule structure and function are based on observations of the properties of brain microtubules. Additionally, the advantage of being able to work with the microtubules of a cultured cell system rather than brain microtubules is that the experiments necessary to verify the association of a presumptive MAP with microtubules in vivo are more readily feasible. Subsequent to this verification, future in vivo experiments to determine the role of associated proteins in microtubule function are also more feasible with a cultured cell system in which cells are growing and dividing than with differentiated, nondividing brain tissue.

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