Identification of a Novel Nucleotide-sensitive Microtubule-binding Protein in HeLa Cells

Janet E. Rickard and Thomas E. Kreis
European Molecular Biology Laboratory, D-6900 Heidelberg, Federal Republic of Germany

Abstract. A protein of Mr 170,000 (170K protein) has been identified in HeLa cells, using an antiserum raised against HeLa nucleotide-sensitive microtubule-binding proteins. Affinity-purified antibodies specific for this 170K polypeptide were used for its characterization. In vitro sedimentation of the 170K protein with taxol microtubules polymerized from HeLa high-speed supernatant is enhanced in the presence of an ATP depleting system, but unaffected by the non-hydrolyzable ATP analogue AMP-PNP. In addition, it can be eluted from taxol microtubules by ATP or GTP, as well as NaCl. Thus it shows microtubule-binding characteristics distinct from those of the previously described classes of nucleotide-sensitive microtubule-binding proteins, the motor proteins kinesin and cytoplasmic dynein, homologues of which are also present in HeLa cells. The 170K protein sediments on sucrose gradients at ~6S, separate from kinesin (9.5S) and cytoplasmic dynein (20S), further indicating that it is not associated with these motor proteins. Immunofluorescence localization of the 170K protein shows a patchy distribution in interphase HeLa cells, often organized into linear arrays that correlate with microtubules. However, not all microtubules are labeled, and there is a significant accumulation of antigen at the peripheral ends of microtubules. In mitotic cells, 170K labeling is found in the spindle, but there is also doty labeling in the cytoplasm. After depolymerization of microtubules by nocodazole, the staining pattern is also patchy but not organized in linear arrays, suggesting that the protein may be able to associate with other intracellular structures as well as microtubules. In vinblastine-treated cells, there is strong labeling of tubulin paracrystals, and random microtubules induced in vivo by taxol are also labeled by the antibodies. These immunofluorescence labeling patterns are stable to extraction of cells with Triton X-100 before fixation, further suggesting an association of the protein with cytoplasmic structures. In vivo, therefore, the 170K protein appears to be associated with a subset of microtubules at discrete sites. Its in vitro behavior suggests that it belongs to a novel class of nucleotide-sensitive microtubule-binding proteins.

INTERPHASE microtubules play important roles in establishment of cell polarity and the organization of the intracellular space (for reviews see Solomon, 1981; Kirschner and Mitchison, 1986), as well as providing tracks for movement of intracellular organelles (Allen et al., 1985; Vale et al., 1985a; reviewed by Schliwa, 1984; Vale, 1987). These functions of microtubules do not depend on the properties of tubulin alone, but are modulated by interactions with other proteins. Proteins defined as microtubule-associated proteins (MAPs) were originally identified in neuronal tissue, an abundant source of microtubules, based on their in vitro microtubule-binding and assembly-promoting activities (reviewed by Olmsted, 1986), but the in vivo role of these proteins has not yet been defined. MAPs from non-neuronal tissues and cultured cells have been more difficult to identify, owing to their lower abundance. It is apparent, however, that there are tissue and species-specific differences in the types of MAPs found in neuronal and non-neuronal cells (reviewed by Wiche, 1989; Matus, 1988; Olmsted, 1986), presumably related to differences in function.

A recently discovered class of proteins involved in microtubule functioning are the microtubule-based mechanochemical enzymes of which two types, kinesin (Mr of major polypeptide, 110,000–120,000) and cytoplasmic dynein (Mr of major polypeptide >400,000), have been described (for reviews see Vale, 1987; McIntosh and Porter, 1989). These proteins are characterized by their nucleotide-sensitive binding to microtubules and microtubule-activated ATPase activity, as well as their ability to induce movement of particles along microtubules in vitro. Because their interaction with microtubules is modulated by nucleotides, these proteins do not satisfy the classical criterion for definition of MAPs, copurification with microtubules during cycles of assembly and disassembly, and are better described as microtubule-binding proteins. Unlike the classical MAPs so far described, there is much evidence for an important role of this class of microtubule-binding proteins in movement of intracellular organelles along

1. Abbreviations used in this paper: AMP-PNP, 5'-adenylimidodiphosphate; MAP, microtubule-associated protein.
microtubules (Schröer et al., 1988, 1989; Schnapp and Reese, 1989). Although kinesin and cytoplasmic dynein were originally isolated from neuronal tissue (Brady, 1985; Vale et al., 1985b; Paschal et al., 1987), they have now been identified in a wide range of tissues and species (reviewed by McIntosh and Porter, 1989) including Drosophila (Saxton et al., 1988), sea urchin eggs (Scholey et al., 1985), the ameba Reticulomyxa (Euteneuer et al., 1988) and the nematode Caenorhabditis elegans (Lye et al., 1987), demonstrating their fundamentally similar role in neuronal and nonneuronal systems.

Kinesin and cytoplasmic dynein induce movement of particles in opposite directions along microtubules, to the plus and minus ends, respectively (Vale et al., 1985c; Paschal and Vale, 1987). These proteins could, therefore, be sufficient to fulfill the cellular requirements for motor proteins able to transport organelles either towards or away from the cell periphery along a unipolar array of microtubules. However, there may exist other motor proteins, possibly specific for particular organelles, and occurring in lower abundance than kinesin and cytoplasmic dynein. A primary characteristic for identification of such novel motors would be nucleotide-sensitive binding to microtubules or a microtubule-activated ATPase activity. A microtubule-activated ATPase from bovine brain of M, 100,000, dynamin, which has properties distinct from kinesin and cytoplasmic dynein has recently been described (Shpeter and Vale, 1989), and sea urchin eggs contain a microtubule-activated ATPase, which, unlike the microtubule-based motor proteins, binds to microtubules in the presence and absence of ATP (Collins and Vale, 1986). These proteins, therefore, may belong to a novel class of microtubule-based mechanochemical enzymes. A lysosome membrane protein of M, 50,000 with ATP-sensitive microtubule-binding properties has also been described (Michieux and Roussel, 1989). Although there is no evidence of mechanochemical activity for this protein, it may be involved in the interaction between lysosomes and microtubules. In addition to the requirement for force-producing enzymes, there is evidence from in vitro experiments that the movement of organelles along microtubules depends on other factors that are not tightly bound to membranes, and may be isolated in association with the motor proteins (Schröer et al., 1988, 1989; Schnapp and Reese, 1989). These "motor-associated proteins" could also show the property of association with microtubules via an interaction with microtubule-based motor proteins, although possibly unable themselves to bind to purified tubulin.

In an attempt to define the molecular mechanisms involved in microtubule-based organelle translocations in nonneuronal cells which have been described in vivo (reviewed by Schliwa, 1984) and in vitro (Dabora and Sheetz, 1988a,b; Vale and Hotani, 1988), we have analyzed HeLa tissue culture cells for the presence of ATP-sensitive microtubule-binding proteins, using approaches developed for the isolation of kinesin and cytoplasmic dynein (Vale et al., 1985b; Amos, 1987; Lye et al., 1987). In addition to proteins homologous to these two motor proteins, we have identified a polypeptide of M, 170,000 (designated 170K protein) which is a consistent component of the crude motor protein extracts. Using an antibody specific for this protein, we have investigated its in vitro binding to microtubules, and its subcellular distribution. The results show that the 170K protein binds in vitro to microtubules in a nucleotide-sensitive manner that is distinguishable from the properties of kinesin and cytoplasmic dynein. In addition, it is localized on microtubules in vivo, but at discrete sites on a subset of microtubules. This protein, therefore, appears to be a novel nucleotide-sensitive microtubule-binding protein.

### Materials and Methods

#### Materials

Taxol was a gift of the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD). Nocodazole (Sigma Chemical Co., Taufkirchen, FRG) and taxol were stored as stock solutions in DMSO at -20°C; vinblastine (Sigma Chemical Co.) was dissolved in H2O and stored at -20°C. Hexokinase (type F-300) and ATP (prepared to be vanadate free) were from Sigma Chemical Co. All other reagents were of analytical grade.

#### Preparation of Bovine Brain Taxol Microtubules

Microtubules were prepared from bovine brain by two cycles of polymerization and depolymerization, and tubulin purified from the microtubule protein by phosphocellulose chromatography as described (Mitchison and Kirschner, 1984). Purified tubulin was polymerized in the presence of 20 μM taxol for 30 min at 37°C, sedimented at 30,000 g for 30 min at 20°C and washed once before resuspension at 5–10 mg/ml in 0.1 M KPipes, 5 mM EGTA, 1 mM MgSO4, pH 6.8. The taxol-stabilized microtubules were stored at -70°C.

#### Preparation of HeLa Cell Proteins

HeLa-S3 cells were grown in suspension in Joklik's MEM supplemented with 1% glucose, 1% penicillin and streptomycin, and 5% newborn calf serum at 37°C in air to densities of 4–6 × 10^5/ml. Cells were harvested at 500 g for 10 min at ambient temperature, washed twice in PBS at 4°C, then once in 0.1 M KPipes, 1 mM MgSO4, 2 mM EGTA, pH 6.8 at 4°C. The washed cell pellet was swollen in 5–10 vol of 1 mM MgSO4, 2 mM EGTA, pH 7.0 at 0°C for 4 min, and then pelleted at 500 g for 10 min at 4°C. The supernatant was removed and the cell pellet (swollen to approximately twice its original volume) made to 0.1 M KPipes, pH 6.8, 1 mM DTT, 1 mM PMSF, and 1 μg/ml each of aprotinin, leupeptin, and pepstatin. The cells were then homogenized by 20–40 strokes in a tight-fitting Dounce homogenizer to achieve >90% cell breakage. The homogenate was centrifuged at 190,000 rpm (40,000 g) in a rotor (model SS-34; Sorvall Div., Norwalk, CT) for 10 min at 4°C, and the low-speed supernatant was centrifuged at 50,000 rpm (150,000 g) in a rotor (model T100; Beckman Instruments, Fullerton, CA) for 90 min at 4°C, to generate a high-speed supernatant extract. The protein concentration of the extract, measured using the Bradford assay (Bradford, 1976) with BSA as a standard, was between 6 and 8 mg/ml. The procedure for isolation of nucleotide-sensitive microtubule-binding proteins from this extract was based on methods used for the isolation of kinesin and cytoplasmic dynein (Vale et al., 1985b; Amos, 1987; Lye et al., 1987). The high-speed supernatant extract was polymerized in the presence of 20 μM taxol at 37°C for 30 min, then centrifuged at 30,000 g for 30 min at 20°C. The supernatant, which is depletes of endogenous tubulin and a large fraction of the MAPs but still contains most of the 170K protein (see Fig. 2), was then incubated with 10 U/ml hexokinase, 10 mM glucose, 30 μM AMP-PNP and bovine brain taxol microtubules at a final concentration of 0.2 mg/ml for 15 min at 37°C, to induce association of the 170K protein with microtubules (Fig. 2). The microtubules were sedimented at 30,000 g for 30 min at 20°C and washed once in wash buffer (0.1 M KPipes, 2 mM EGTA, 1 mM MgSO4, 2 mM DTT, 20 μM taxol, pH 6.8). Proteins were eluted from the microtubules by resuspension in wash buffer (usually 0.1 to 0.05 times the original high-speed supernatant volume) containing 10 mM MgATP, 10 mM MgGTP, or 0.5 M NaCl, and incubation at 37°C for 10 min followed by centrifugation.

ATP-eluted proteins were further fractionated by sucrose gradient centrifugation (Lye et al., 1987; Paschal et al., 1987). Extracts (0.4 ml) were loaded onto 4-ml gradients of 5–25% sucrose in wash buffer and centrifuged at 33,000 rpm (100,000 g) in a rotor (model SW60; Beckman Instruments) for 16 h at 4°C. 20 fractions of 0.2 ml were collected from the top.
Gel Electrophoresis and Immunoblotting

Reduced and alkylated proteins were separated by SDS-PAGE according to Laemmli (1970), with a 7% acrylamide running gel and a 3% acrylamide stacking gel, using 0.75 mm thick mini-gels (Bio-Rad Laboratories GmbH, München, FRG) run at 25 mA. Gels were stained with Coomassie brilliant blue, or silver using the method of Heukeshoven and Dernick (1988). Molecular mass standards were from Bio-Rad Laboratories. For immunoblotting, proteins were transferred to nitrocellulose paper (0.2 μm pore size; Schleicher & Schuell Co., Dassel, FRG) using a Genie electrophoreser (Ideo Scientific Co., Corvallis, OR) with 25 mM Tris, 192 mM glycine at 24 V for 30 min with cooling. Excess binding sites on the filters were blocked with PBS containing 5% dried nonfat milk powder and 0.2% Triton X-100 (blocking buffer) before incubation with antibody, diluted in the same buffer, for 16-24 h at 4°C. Bound antibody was visualized using alkaline phosphatase-conjugated second antibody as described (Allan and Kreis, 1986).

Production and Affinity Purification of Antibodies

ATP-sensitive microtubule-binding proteins were prepared from HeLa cells for use as immunogen as described above; proteins were eluted with both ATP and NaCl as these conditions gave the highest yield of protein. This resulted in the presence of some non-nucleotide sensitive MAPs in the extract (see Fig. 3), one of which, the 210K MAP (Bulinski and Borisy, 1979; Weatherbee et al., 1980), also consistently produced an immune response (see Discussion). For immunization of mice, the eluate (protein concentration ~50 μg/ml) was emulsified into an equal volume of complete (for first immunization) or incomplete (for subsequent immunizations) Freund's adjuvant (Gibco Laboratories, Grand Island, NY) and 200-μl aliquots used for subcutaneous injection in BALB/c mice. The animals were boosted 3-4 wk after the first immunization, and subsequently after a minimum of 4 wk. Test bleeds were taken 10 d after each boost, and after two to three boosts the animals were sacrificed to obtain ~0.5 ml serum per animal. Rabbits were immunized following the protocol of Louvard et al. (1982), using ~0.6 ml eluate per injection. Antibodies specific for the 170K protein were affinity purified from nitrocellulose blots by a modification of the method of Olimsted (1981) and Hurt et al. (1988). A taxol pellet prepared from 4 ml HeLa high-speed supernatant was electrophoresed on a preparative 5% polyacrylamide gel (1.5 mm thick, 17 × 9 cm) and blotted onto nitrocellulose paper as described (Allan and Kreis, 1986) using the buffer of Bloom and Vallee (1983). The 170K protein was located by immunolabeling of strips cut from either side of the blot, and then cut out from the rest of the blot. After blocking of nonspecific binding sites, strips were incubated with antiserum diluted in blocking buffer for 16-24 h at 4°C. The strips were washed for 30 min in three changes of PBS, rinsed briefly in distilled water, and the antibodies eluted for 3 min in 1 ml of 50 mM glycine, 0.5 M NaCl, pH 2.8, followed by 3 min in 1 ml of the same buffer at pH 2.2. Eluted antibodies were neutralized by addition of 0.11 vol of 0.5 M Na2HPO4, and BSA was added to a final concentration of 0.25 mg/ml. After washing the strips in PBS, they were reincubated with the diluted antisera for 7 h at 4°C, followed by washing and elution as before. The pooled eluates were concentrated and the buffer changed to PBS using a Centricon 30 (Amicon Corp., Danvers, MA). The antibodies were stored at 4°C before use at appropriate dilutions for immunofluorescence and immunoblotting. Both rabbit and mouse antibodies were used for the experiments reported here with no significant difference in results.

Immunofluorescence

HeLa cells were grown on coverslips in MEM supplemented with 1% nonessential amino acids, 1% glutamine, 1% penicillin and streptomycin and 10% FCS in humidified 5% CO2 incubators (7% CO2, 93% air) at 37°C. Cells were fixed for 4 min each in methanol followed by acetone at -20°C, in some cases after preextraction in 80 mM KPi, 5 mM EGTA, 1 mM MgCl2, 0.5% Triton X-100, pH 6.8 as described (Kreis, 1987). Fixed cells were labeled with affinity-purified mouse or rabbit antibodies to the 170K protein and mouse monoclonal or rabbit polyclonal antibodies specific for tyrosinated tubulin (1A2 of α-T13, respectively; Kreis, 1987) followed by rhodamine or fluorescein-labeled secondary antibodies (Allan and Kreis, 1986). Epifluorescence microscopy was performed using a Zeiss 63× Planapo, 1.4 oil immersion objective on a Zeiss Axiovert microscope, and images were recorded on Kodak T-Max P3200 film.

Figure 1. ATP-sensitive microtubule-binding proteins of HeLa cells, and the specificity of the affinity-purified antibodies to the 170K protein. A low-speed supernatant extract (lanes 1 and 4), high-speed supernatant extract (lanes 2 and 5), and ATP-eluted nucleotide-sensitive microtubule-binding proteins (lanes 3 and 6) were prepared from HeLa cells as described in Materials and Methods and separated by SDS-PAGE followed by staining with Coomassie brilliant blue (lanes 1 and 2) or silver (lane 3), or immunoblotting with affinity-purified anti-170K (lanes 4-6). The positions of the major polypeptides of cytoplasmic dynein (D) and kinesin (K) are indicated. Molecular weights are indicated on the right in kilodaltons. Material loaded in lanes 4 and 5 was derived from equivalent amounts of cells to allow comparison on the immunoblot of the recovery of the 170K protein in the high-speed supernatant.

Figure 2. Nucleotide-sensitive sedimentation of the 170K protein with microtubules. A HeLa high-speed supernatant extract was polymerized at 37°C for 30 min in the presence of 20 μM taxol with the additions indicated, and the microtubules were sedimented at 30,000 g for 30 min through a cushion of 10% sucrose in wash buffer at 20°C. The pellets and an equivalent volume of the high-speed supernatant were then analyzed by SDS-PAGE and immunoblotting with the antibodies to the 170K protein. (A) silver-stained gel and (B) immunoblot of (1) total high-speed supernatant, and pellets from: (2) no addition; (3) 5 mM AMP-PNP; (4) 10 μM ATP; (5) 1 mM MgATP. The position of the 120K polypeptide of kinesin (K) is indicated on the stained gel by dots. Molecular weights are indicated on the right in kilodaltons.
Results

ATP-sensitive Microtubule-binding Proteins of HeLa Cells

Nucleotide-sensitive microtubule-binding proteins were prepared from HeLa cells as described in Materials and Methods, using a microtubule-affinity purification approach which was previously applied for the isolation of kinesin (Vale et al., 1985b; Amos, 1987) and cytoplasmic dynein (Lye et al., 1987). The ATP-eluted proteins were analyzed by SDS-PAGE (Fig. 1, lane 3). Major polypeptides of M, 120,000 and >300,000 are present in this extract, which was found to support gliding of microtubules over glass (data not shown). The 120K protein has been identified as kinesin based on its AMP-PNP-dependent binding to microtubules and its immunological cross-reactivity with an antibody to squid optic lobe kinesin (rabbit polyclonal antibody kindly provided by Dr. M. P. Sheetz, University of St. Louis, data not shown). The high molecular weight polypeptide sediments at ~20S on sucrose gradients; after ion exchange chromatography of the sucrose gradient-purified material (data not shown) it has identical relative molecular mass and polypeptide composition (heavy and light chains) to calf brain MAP1C (Paschal et al., 1987; Paschal and Vallee, 1987) and has therefore been identified as cytoplasmic dynein. The presence of these motor proteins in the ATP eluate is not unexpected, as they appear to be widely distributed, and have now been purified from several nonneuronal tissues and cultured cell lines.

In addition to kinesin and cytoplasmic dynein, several minor polypeptides (present at less than 10% of the concentration of kinesin) are present in this extract, some of which appear to be specifically eluted by ATP (Fig. 1, lane 3). Polyclonal antibodies were raised against this crude extract and antibodies to a polypeptide of M, 170,000 (170K protein) were affinity purified from nitrocellulose blots of taxol alone and AMP-PNP, to bind the 170K protein. (A) Silver-stained gel and (B) immunoblot of: (1, 3, 5, and 7), supernatants and (2, 4, 6, and 8), pellets after incubation of the microtubules with (l and 2) wash buffer alone; (3 and 4) 10 mM MgATP; (5 and 6) 10 mM MgGTP; (7 and 8) 0.5 M NaCl. The positions of cytoplasmic dynein (D), kinesin (K), and the 210K HeLa MAP (M) are indicated on the stained gel by dots. Molecular weights are indicated on the right in kilodaltons.

Figure 3. Elution of the 170K protein from microtubules by GTP and ATP. Nucleotide-sensitive microtubule-binding proteins were prepared from HeLa cells as described in Materials and Methods. Tubulin and MAPs were depleted from a high-speed supernatant by taxol polymerization. Bovine brain taxol microtubules were added to the supernatant, together with an ATP-depleting system and AMP-PNP, to bind the 170K protein and motor proteins. The microtubules were washed, then eluted with ATP, GTP, or NaCl as indicated, and equivalent amounts of the supernatants and pellets analyzed by SDS-PAGE and immunoblotting for the 170K protein. (A) Silver-stained gel and (B) immunoblot of: (l, 3, 5, and 7), supernatants and (2, 4, 6, and 8), pellets after incubation of the microtubules with (l and 2) wash buffer alone; (3 and 4) 10 mM MgATP; (5 and 6) 10 mM MgGTP; (7 and 8) 0.5 M NaCl. The positions of cytoplasmic dynein (D), kinesin (K), and the 210K HeLa MAP (M) are indicated on the stained gel by dots. Molecular weights are indicated on the right in kilodaltons.

Sedimentation of the 170K Protein with Microtubules Is Nucleotide Sensitive

Kinesin is known to bind efficiently to microtubules only in the presence of AMP-PNP, whereas sedimentation of cytoplasmic dynein with microtubules occurs simply in the absence of ATP (Brady, 1985; Scholey et al., 1985; Vale et al., 1985b; Paschal et al., 1987). The presence of the 170K protein in the HeLa ATP extract containing kinesin and cytoplasmic dynein suggested that its interaction with microtubules may also be nucleotide-sensitive. The characteristics of this binding were investigated by polymerizing a high-speed supernatant extract of HeLa cells in the presence of taxol, together with AMP-PNP, an ATP-depleting system, or ATP. The microtubule pellets were analyzed by SDS-PAGE and immunoblotting (Fig. 2). The total high-speed supernatant content of 170K protein revealed by immunoblotting is shown in Fig. 2, lane 1'. Microtubules sedimented after incubation with taxol alone contain only a small fraction of the total 170K protein (Fig. 2, lane 2'), and this pellet-associated 170K protein is decreased in the presence of 1 mM ATP (Fig. 2, lane 3'). Polymerization of the extract in the presence of 5 mM AMP-PNP has no effect on the amount of 170K pro-
tein sedimenting with the pellet (Fig. 2, lane 3'). This amount of AMP-PNP is known to enhance association of kinesin with microtubules (Brady, 1985; Scholey et al., 1985; Vale et al., 1985b), and indeed causes increased sedimentation of the 120K polypeptide we have identified as kinesin (Fig. 2, lane 3). In contrast, addition of an ATP-depleting system during the taxol polymerization causes >50% of the 170K protein to pellet (Fig. 2, lane 4'). The increase in sedimentation of the 170K protein with microtubules after ATP depletion, together with the decreased association in the presence of ATP, suggest an effect of ATP on a specific interaction of this protein with microtubules. The conditions for elution of the 170K protein from microtubules were, therefore, investigated.

The 170K Protein Is Eluted from Microtubules by ATP and GTP

Kinesin and cytoplasmic dynein can be eluted from microtubules by ATP, but differ in that kinesin is also released by GTP, whereas binding of cytoplasmic dynein is insensitive to this nucleotide (Paschal et al., 1987). To investigate a possible relationship of the 170K protein to kinesin and cytoplasmic dynein, its elution efficiency with ATP and GTP was tested. Nucleotide-sensitive microtubule-binding proteins were prepared from a microtubule-depleted HeLa high-speed supernatant extract as described in Materials and Methods. The microtubule pellet was eluted with nucleotides or NaCl, and the supernatants and pellets analyzed by immunoblotting with the 170K antibodies. After washing of the microtubule pellet in control buffer, very little protein appears in the supernatant (Fig. 3, lane J), and there is no 170K protein detectable by immunoblotting (Fig. 3, lane J'). In contrast, significant amounts of the 170K protein are eluted by ATP (Fig. 3, lane J'), GTP (Fig. 3, lane 5') or NaCl (Fig. 3, lane 7'). The corresponding stained gel demonstrates that, as expected (Paschal et al., 1987), kinesin is eluted by both GTP and ATP, whereas cytoplasmic dynein is eluted only by ATP (Fig. 3, compare lanes 3 and 5). Both these motor proteins are also eluted by NaCl (Fig. 5, lane 7). The selectivity of the nucleotide elution is demonstrated by the insensitivity of a polypeptide of Mr ~200,000 present in the microtubule pellet, which we assume to be the 210K HeLa MAP (Bulinski and Borisy, 1979; Weatherbee et al., 1980), to elution by nucleotides; it can, however, be washed off by NaCl (Fig. 3, lane 7). The proportion of 170K protein released by the nucleotides varied slightly between experiments. However, ATP consistently released more 170K protein (~50%) than GTP, but less than NaCl. Further protein could be released in a second nucleotide wash, but the reason for the incomplete elution in a single wash is not clear. It is not simply due to insufficient nucleotide, as a 10-fold lower ATP concentration (1 mM) releases a similar amount of the 170K protein as 10 mM (not shown). Kinesin is also reported to be only partially eluted from microtubules by a single ATP wash (Vale et al., 1985b; Paschal et al., 1987). These data further indicate a nucleotide-specific association of the 170K protein with microtubules. Its lack of dependence on AMP-PNP for binding (Fig. 2), together with its sensitivity to elution by GTP (Fig. 3) distinguish it from both kinesin and cytoplasmic dynein and suggest that its association with microtubules is independent of these motor proteins.

Neely and Boekelheide (1988) reported a polypeptide of Mr, 173,000 cosedimenting on sucrose gradients at 20S with cytoplasmic dynein isolated from rat Sertoli cells, and Schroer et al. (1989) described a polypeptide similarly associating with cytoplasmic dynein from chick embryo brain and fibroblasts, but estimated an Mr of 154,000. To examine a possible relationship of the 170K protein described here to these reported dynein-associated proteins, we fractionated the extract of HeLa proteins released from microtubules by ATP on sucrose gradients. The 170K protein sediments at ~6S (Fig. 4), well separated from cytoplasmic dynein (20S; Lye et al., 1987; Paschal et al., 1987) as well as kinesin (9.5S; Bloom et al., 1988). A polypeptide of calculated Mr, 150,000 cosediments with cytoplasmic dynein in our extract, and is presumably equivalent to the previously reported proteins mentioned above (Neely and Boekelheide, 1988; Schroer et al., 1989). We found that this 150K polypeptide could be separated from cytoplasmic dynein by ion exchange chromatography, suggesting that it is not a subunit of the motor protein (data not shown). This sedimentation behavior of the 170K protein (Fig. 4) further indicates that its in vitro microtubule binding characteristics are not due to a close association with either of the two known motor proteins.

Immunofluorescence Colocalization of the 170K Protein with Microtubules

The affinity-purified antibodies were used for indirect immunofluorescence localization of the 170K protein in HeLa cells. In interphase cells, the 170K antibodies label patchy structures that appear to be organized into linear arrays (Fig. 5a), and may be coincident with the pattern of microtubule

Figure 4. Separation of ATP-sensitive microtubule-binding proteins from HeLa cells by sucrose gradient centrifugation. ATP-eluted microtubule-binding proteins from HeLa cells were separated on an 5–25% sucrose gradient as described in Materials and Methods. Fractions from the gradient separated by SDS-PAGE were analyzed by silver staining (A) and immunoblotting for the 170K protein (B). S, starting sample; 1–17, fraction number. The positions of cytoplasmic dynein (D), kinesin (K), and the 170K protein (170K) are indicated on the stained gel by dots. Molecular weights are indicated on the right in kilodaltons.
staining (Fig. 5 b). In addition, this patchy labeling is stable to detergent extraction before fixation (compare Figs. 5, a and c), suggesting that the antigen is to a large extent linked to an intracellular structure. The labeling pattern for the 170K protein was stable to extraction for times of up to 4 min, sufficient to remove soluble tubulin completely (data not shown). However, it was consistently found that detergent preextraction led to a clearer image of the discrete structures labeled by the antibodies, suggesting that there may also be a significant soluble cytoplasmic pool of the protein.

Examination of regions close to the cell edge, where single microtubules can be distinguished, shows overlap between the 170K antigen and the position of microtubules. Interestingly, the protein is found on only some of the microtubules and, where single microtubules can be unambiguously traced for some distance, there is a significant accumulation of labeling at microtubule ends (Fig. 5, c and d, arrowheads; arrows indicate microtubules negative for the 170K protein). This immunofluorescence localization indicates an in vivo association of the 170K protein with microtubules, in addition to its in vitro binding properties. However, the protein does not have the uniform distribution along microtubules described for other MAPs (Bulinski and Borisy, 1980a; Bloom et al., 1984; reviewed by Olmsted, 1986), but appears concentrated at discrete sites.

**Localization of the 170K Protein in the Mitotic Spindle**

In mitotic HeLa cells, the antibodies to the 170K protein clearly label the mitotic spindle (Fig. 6, a and c), further suggesting an association of the 170K protein with microtubules, although this cannot be unambiguously shown at the level of the light microscope. There is also punctate labeling of the mitotic cytoplasm, which does not colocalize with
microtubules. This indicates an association of the 170K protein with discrete cytoplasmic structures; these structures are stable to detergent extraction before fixation (not shown). Thus, although the 170K protein localizes in the mitotic spindle, there is also some labeling not associated with microtubules.

The Effect of Microtubule-active Drugs on the Distribution of the 170K Protein

The localization of the 170K protein in interphase and mitotic cells strongly suggests an in vivo association of the protein with microtubules. The effect of microtubule-active drugs was, therefore, investigated. Depolymerization of the interphase microtubules with nocodazole disrupts the patchy arrays of the 170K protein characteristically found in untreated cells. The antigen does not, however, become diffuse, as does the tubulin, but appears associated with discrete structures in the cytoplasm (Fig. 7, a and b). The nocodazole-treated cells also contain a few filaments which are strongly labeled by the antibodies to the 170K protein (Fig. 7 a, large arrowheads); examination of cells extracted with detergent before fixation to remove soluble tubulin shows that these are microtubules (data not shown). The association of the 170K protein along the length of these remaining microtubules is in contrast to the apparent preferential end localization found in untreated cells (Fig. 5, a and c), and indicates that the 170K protein is not exclusively an end-associated protein. The labeling pattern of nocodazole-treated cells is unaffected by detergent extraction of the cells before fixation (not shown), suggesting that the 170K protein associates with another intracellular structure in the absence of microtubules. This observation is apparently inconsistent with the fact that the majority of the 170K protein is present in the soluble cytoplasmic extract during cell fractionation. The reason for this is not clear, but presumably the homogenization conditions employed more efficiently solubilize the protein than the brief detergent extraction used before fixation. In contrast to these effects of nocodazole, disruption of the actin filament network after incubation of cells with 1 μM cytochalasin D for 1 h at 37°C had no effect on the co-distribution of the 170K protein with microtubules (data not shown), indicating no involvement of microfilaments in its organization (see also Discussion). Intermediate filaments are known to colocalize and interact with microtubules, and their organization is affected by nocodazole treatment (Singer et al., 1981; Geuens et al., 1983). However, a primary association of the 170K protein with intermediate filaments is unlikely, since the short-term nocodazole treatment used here, which disrupts the organization of the 170K protein, would not be expected to affect significantly the intermediate filament network (Hyne and Destree, 1978; Lazarides, 1978). In addition, depolymerization of microtubules by cold treatment, which does not lead to disruption of the intermediate filament network (Virtanen et al., 1980), also destroyed the patchy arrays of the 170K protein (data not shown).

Treatment with vinblastine induces tubulin paracrystals (Bensch and Malawista, 1968) which label strongly for the 170K protein, indicating a high affinity of the protein for microtubules (Fig. 7, c and d). Taxol, added during depolymerization of microtubules after removal of nocodazole, induces formation of random short tubulin polymers in the cytoplasm, some of which are heavily labeled by the antibodies to the 170K protein (Fig. 7, e and f, small arrowheads), although there is also significant cytoplasmic staining for the 170K protein which does not appear to be microtubule-associated. The effects of these microtubule-active drugs on the organization of the 170K protein further indicate an in vivo association of this protein with microtubules. However, the patchy distribution of the protein after nocodazole treatment, together with its resistance to detergent extraction, suggests that it may also associate with other intracellular structures, as was also indicated from the labeling pattern found in mitotic cells (Fig. 6).

Discussion

Cytoplasmic dynein and kinesin are mechanochemical enzymes, which are most likely involved in microtubule-dependent intracellular organelle transport (reviewed by Vale, 1987; McIntosh and Porter, 1989). In addition to these two motor proteins, the existence of other essential factors has been inferred from studies of components involved in movement of organelles along microtubules in vitro (Schroer et al., 1988, 1989; Schnapp and Reese, 1989). These factors appear to have motor-binding activity, and can be postulated to form part of a translocator complex (Schroer et al., 1988). So far, none of these additional factors has been identified. Furthermore, microtubule-dependent motor proteins different from cytoplasmic dynein and kinesin may exist.

We describe here a novel protein in HeLa cells of Mr 170,000 (170K protein) which associates with microtubules.
Figure 7. Distribution of the 170K protein after treatment with microtubule-active drugs. HeLa cells were incubated with (a and b) 10 μM nocodazole for 1 h at 37°C; (c and d) 10 μg/ml vinblastine for 3 h at 37°C; (e and f) 10 μM nocodazole for 1 h at 37°C, followed by 15 min at 0°C in drug-free medium, then 10 μM taxol for 60 min at 37°C, before fixation as described in Materials and Methods and double immunofluorescence labeling for the 170K protein (a, c, and e) and tubulin (b, d, and f). The 170K protein was labeled with rabbit (a and e) or mouse (c) antibodies. Bar, 20 μm.
in vitro in a nucleotide-sensitive manner, and colocalizes with microtubules by immunofluorescence. The interaction of the 170K protein with microtubules is affected by nucleotides, but the characteristics of its association make it distinguishable from the two previously characterized nucleotide-sensitive microtubule-binding proteins. Kinesin, but not the 170K protein, binds efficiently to microtubules only in the presence of AMP-PNP (Brady, 1985; Scholey et al., 1985; Vale et al., 1985b; see also Fig. 2), and HeLa cytoplasmic dynein can be eluted from microtubules only by ATP, whereas the 170K protein dissociates from microtubules in the presence of ATP and GTP. In addition, the 170K protein sediments on sucrose gradients at ~6S, well separated from kinesin (9.5S; Bloom et al., 1988) and cytoplasmic dynein (20S; Lyer et al., 1987; Paschal et al., 1987). Binding of the 170K protein to tubulin may either be direct or indirect via nucleotide-sensitive microtubule-binding protein(s).

A protein of this relative molecular mass has not been described previously in studies of HeLa microtubule proteins (Bulinski and Borisy, 1979; Weatherbee et al., 1980). These two studies analyzed HeLa cell extracts by classical polymerization and depolymerization cycles, and identified major MAPs of M<sub>r</sub> 200,000-210,000 and 100,000-120,000 (Bulinski and Borisy, 1979; Weatherbee et al., 1980), and minor proteins of M<sub>r</sub> 68,000 and 151,000 (Weatherbee et al., 1980). However, the sensitivity of the 170K protein described here to elution from microtubules by GTP and ATP means that it would be unlikely to remain associated with microtubules through several cycles of polymerization in the presence of nucleotides. A nucleotide-sensitive microtubule-binding protein of this molecular weight has not been previously reported in any species, and the antibodies described here have so far been found to react only with cells of primate origin (human and monkey). This lack of antibody cross-reaction with other species would be consistent with high immunogenicity of the protein, which induced a strong antigenic response although it was a minor component of the extract used for immunization. It is noteworthy that the only other protein in this extract that consistently induced a strong immune response was the 210K HeLa MAP. Polyclonal antibodies against the 210K MAP are also specific for primate cells (Bulinski and Borisy, 1980b), although an mAb to this protein cross-reacting with marsupial cells has been described (Izant et al., 1983). Homologues of this MAP have, however, been found in several species (Parysek et al., 1984; Goldstein et al., 1986; Kotani et al., 1988). Proteins homologous to the HeLa 170K protein may, therefore, also be widely distributed.

The immunofluorescence localization of the 170K protein distinguishes it from previously described MAPs, as well as kinesin. MAPs have been commonly shown to localize on all microtubules along their entire length. This is true both for neuronal MAPs (reviewed by Vallee and Bloom, 1984; Olmsted, 1986), and for the 210K and 125K MAPs isolated from HeLa cells (Bulinski and Borisy, 1980a). An exception to this is an antibody to MAP1A, which gave punctate labeling of interphase microtubules (Bloom et al., 1984). However, in the case of this MAP, labeling was periodic along the entire length of all the microtubules, and there was no labeling of vinblastine paracrystals (Bloom et al., 1984). Kinesin has been localized by immunofluorescence to various structures, including mitotic spindles (Scholey et al., 1985), the centrosome and spindle poles (Neighbors et al., 1988), interphase microtubules and the cytoplasmic matrix (Murofushi et al., 1988; Hollenbeck, 1989), and vesicular structures assumed to be intracellular organelles (Pfister et al., 1989). The staining pattern for the 170K protein described here does not correspond to any of these reported localizations for kinesin, which further suggests that kinesin and the 170K protein are not associated. In addition, staining with antibodies against kinesin is sensitive to Triton X-100 extraction (Hollenbeck, 1989; Pfister et al., 1989), which is not the case for the labeling for the 170K protein. There are so far no reported immunolocalizations of cytoplasmic dynein.

The nucleotide-sensitive microtubule association of the 170K protein suggests that it may be a novel motor or motor-associated protein, in which case its localization may indicate sites of interaction between a subclass of microtubules and organelles. It has been suggested previously that movement of organelles occurs along a subset of microtubules and organelles. However, the presence of the majoritiy of the 170K protein in the soluble cell extract indicates that it is not tightly associated with membranes, at least under the extraction conditions used here. A possible colocalization of the 170K protein with organelles and microtubules could best be investigated by immunoelectron microscopy, as well as biochemical analysis of isolated membrane fractions. Electron microscopical studies have not so far been attempted, mainly because the antigen detected using these antibodies is sensitive to aldehyde fixation. Efforts are currently being made to raise polyclonal antibodies to fusion proteins from cDNA clones of the 170K protein expressed in bacteria, and produce mAbs, to facilitate further biochemical and immunocytochemical studies.

Alternative interpretations of the localization of the 170K protein are also possible. For example, the specific location of the 170K protein at the peripheral ends of microtubules could be indicative of sites of attachment of microtubules with the plasma membrane. Rinnerthaler et al. (1988) have presented evidence for a role of microtubules in the definition of sites of cell-substrate interaction leading to formation of more stable contact sites involving actin-containing stress fibers. However, examination of cells labeled by double immunofluorescence for actin and the 170K protein did not provide any conclusive indication of whether or not the 170K protein may be associated with the ends of stress fibers (data not shown). Further investigation of this point will require the resolution of electron microscopy. Moreover, microtubules are dynamic polymers in vivo (Kirschner and Mitchison, 1986), and an association of the 170K protein with their plus ends may play a role in modulating their dynamic properties. The observed interaction of the 170K protein with only some interphase microtubules would be consistent with this interpretation; its selective distribution may indicate a role in defining or stabilizing a subclass of microtubules which differs in its dynamic properties (Kirschner and Schulze, 1986). Clearly, further experiments are necessary to characterize the biochemical properties and the precise cellular function of the 170K protein.

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References


