

# Immunofluorescence Localization of HeLa Cell Microtubule-associated Proteins on Microtubules In Vitro and In Vivo

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**ABSTRACT** Rabbit antisera were prepared against the two major groups of microtubule-associated proteins (MAPs) from HeLa cells, proteins of ~210,000 molecular weight (210k MAPs), and 125,000 mol wt (125k MAPs). These antisera were characterized by a sensitive antigen detection technique that employs immunofluorescence to localize cross-reactive material in polyacrylamide gels. Antisera prepared against the 210k MAPs showed no cross-reactivity with extract proteins of other molecular weights or with brain MAPs, but did react with proteins of 210,000 mol wt and with a minor HeLa MAP of ~255,000 mol wt. Antibodies prepared against the 125k HeLa MAPs, likewise, reacted specifically with proteins of 125,000 mol wt, showing no cross-reactivity with other HeLa extract proteins or porcine brain MAPs. Immunofluorescence with the 210k and 125k MAP antisera was used to demonstrate the association of each of the MAPs with fixed HeLa microtubules in vitro. In addition, immunofluorescence with these antisera revealed a physical association of 210k and 125k MAPs with a Colcemid-sensitive fiber network in fixed interphase and mitotic HeLa cells. Thus, using specific, well-characterized antisera to the two major groups of HeLa MAPs, we have shown that these proteins are components of microtubules in HeLa cells.

Microtubule-associated proteins (MAPs) are minor components of the microtubule protein isolated from vertebrate brain tissue by temperature-dependent self-assembly methods. This class of proteins includes species of very high molecular weight called either HMW (280,000 mol wt [17]) or MAP<sub>2</sub> (300,000 mol wt [28]) and a group of four proteins of 55,000–62,000 mol wt called tau protein (31). Both of these groups of MAPs bind to, copurify with, and stimulate polymerization of, microtubules in vitro (6, 17, 18, 22, 26–28, 31). The HMW MAP has been shown to be responsible for projections on the wall of microtubules by electron microscope analyses (1, 10, 13, 17). However, proteins that specifically associate with tubulin in vitro do not necessarily interact with microtubules in vivo. Molecules that happen to have a high affinity for tubulin under certain solution conditions may adventitiously associate with tubulin in cell extracts and may nonspecifically stimulate microtubule polymerization in vitro. Lee et al. (15) have pointed out that if high affinity binding molecules were present in limiting amounts, they would appear to copurify stoichiometrically with tubulin upon multiple cycles of assembly-disassembly and would be erroneously identified as MAPs. Other

workers (7, 29) have also noted the potential for artifactual association in vitro.

A resolution of this problem is potentially available by immunocytochemical methods. Antibody localization of MAP molecules along microtubules in intact cells would provide evidence for MAP-tubulin interactions in vivo. Several reports of indirect immunofluorescence localization of tau (8) and HMW (9, 23–25) have suggested that these proteins do associate with microtubules in certain cultured cells; however, these reports have certain limitations. In no case have the antisera to tau or HMW been characterized adequately. The specificity of antibodies has not been demonstrated, and a cross-reacting molecule in cell extracts that accounts for the antibody staining in fixed cells has not been identified. Furthermore, purification of microtubules from cells shown to contain tau or HMW by immunofluorescence studies has yielded either no proteins resembling tau or HMW (19, 33, 34) or else amounts too miniscule to characterize adequately (7, 32). Preliminary studies from this laboratory (21) using antibodies to pig brain HMW have failed to detect cross-reactive material in several cultured cell lines. Thus, we suspect that if MAPs similar to

brain HMW do exist in cultured cells, they must be present at very low levels.

We have approached the problem of MAPs in cultured cells by a combination of direct biochemical isolation of binding proteins and immunocytochemical localization using antibodies prepared against the homologous molecules.

Microtubule protein isolated from HeLa cells by *in vitro* polymerization of cell extracts is composed of tubulin and a small amount (5%) of microtubule-associated proteins (MAPs) unlike those isolated from vertebrate brain tissue (2). The MAP species fall into two major groups when analyzed on acrylamide gels: a triplet of bands centered at ~210,000 mol wt and a species of ~125,000 mol wt.

Although both groups of MAPs stimulate polymerization of microtubules *in vitro* (2), the 210k and 125k species behave as independent molecular species and can be separated from each other by sedimentation and molecular sieve chromatography.<sup>1</sup> We have also shown that these proteins can be isolated from mitotic and interphase HeLa cells in the same approximate proportions relative to tubulin (4).

To learn about the interactions of HeLa MAPs with microtubules *in vivo*, we have prepared antibodies to both the 210k and 125k species individually. In this report, we document the specificity of the antisera obtained, using an immunofluorescent staining technique for the detection of antigens in polyacrylamide gels. With these antisera we demonstrate the binding of each of the MAPs to HeLa microtubules polymerized *in vitro*. Using indirect immunofluorescent staining of fixed HeLa cells, we examine the distribution of 210k and 125k MAPs in interphase and mitotic cells and show that the putative MAPs are indeed associated with microtubules *in vivo*.

## MATERIALS AND METHODS

### Preparation of Antisera against HeLa MAPs

HeLa cell microtubule protein was prepared according to Bulinski and Borisy (2) and was electrophoresed on preparative slab gels (3.0 mm thick) containing 6.25% acrylamide. Gels were prepared according to Laemmli (14), stained with Coomassie Brilliant Blue R-250 according to Fairbanks et al. (12), and stored in 10% acetic acid. Stained protein bands were excised from gels with a razor blade, and gel pieces were rinsed in distilled water three times for 30 min to remove acetic acid. Application of 2 mg of HeLa microtubule protein to each preparative gel yielded gel pieces containing 50  $\mu$ g of 210k MAP and pieces containing 44  $\mu$ g of 125k MAP. Gel pieces were placed in an equal volume of an elution solution containing 0.1% SDS and 0.05%  $\beta$ -mercaptoethanol and sonicated at setting 7 with a Heat Systems Sonifier (Branson Ultrasonics, Plainview, N. Y.) for several minutes, until a homogeneous, viscous solution was obtained. This material, 20–50  $\mu$ g MAP in 0.5 ml, was mixed 1:1 with Freund's complete adjuvant (Grand Island Biological Co., Grand Island, N. Y.), and emulsified by passing between two syringes connected by a double-butted needle, or by further sonication. Preimmune sera from female, adult New Zealand White rabbits (Klubertanz, Inc., Edgerton, Wis.) were tested to ensure that they did not contribute background in our experiments. Rabbits were injected with 1 ml of MAP-adjuvant suspension, subcutaneously, in the back fat pad or in the footpads. Rabbits immunized with 210k MAP received three injections, 20–50  $\mu$ g of 210k MAP each, 10 d to 2 wk apart, followed by a booster 2–3 wk later of 50–100  $\mu$ g 210k MAP. Antibodies against 210k MAP were detectable ~2 wk after the first injection by staining of cells and 1 wk after the second injection by staining of gels (see below). Rabbits were bled 40–75 ml each week after the booster injection for ~20 wk. All five of the rabbits injected with 210k MAP produced antisera that reacted strongly with 210k MAP. Rabbits immunized with 125k MAP received three shots of 50  $\mu$ g of 125k MAP each, spaced 10 d to 2 wk apart, and three booster shots of 100–300  $\mu$ g of 125k MAP spaced 3–5 wk apart. Of three rabbits injected with 125k MAP, all three produced antisera that reacted with a Colcemid-sensitive fiber network in fixed cells, but only two produced antibodies

<sup>1</sup> Bulinski, J. C., and G. G. Borisy. 1980. Microtubule-associated proteins from cultured HeLa cells: analysis of molecular properties and effects on microtubule polymerization. *J. Biol. Chem.* In press.

that were detectable by staining of gels. The animals were bled 40–75 ml/wk after detection of 125k antibodies.

Antitubulin IgG was a gift from Dr. Brian S. Spooner and Carter Holladay, Kansas State University.

### Indirect Immunofluorescent Staining of Polyacrylamide Gels

SDS polyacrylamide slab gels, 0.75 mm thick, containing 6–7.5% acrylamide (depending on the experiment) were prepared according to Laemmli (14). Two identical 5 × 7 inch gels (or two halves of a single gel) were prepared, and one gel was stained with Coomassie Brilliant Blue and photographed with Polaroid 665 P/N film. Tubulin and MAP proportions were quantitated using the methods described elsewhere.<sup>1</sup> The other gel was stained with the appropriate MAP antiserum using a modification of the technique of Olden and Yamada (20). Whereas they used anti-immunoglobulin conjugated to horseradish peroxidase and reaction with diaminobenzidine or Coomassie Brilliant Blue to localize the antigen, we have employed a rhodamine-conjugated anti-immunoglobulin and fluorescence. In our procedure, the gel was first soaked in 50 vol methanol:acetic acid (25:10%, vol/vol) to fix the protein. It was then rinsed at least six times, 6 h each, at 55°C, to remove the SDS quantitatively, as demonstrated previously (20). To remove the acetic acid and methanol, the gel was soaked in 50 vol distilled water four times for 30 min. All rinses and incubations from this step on were done at room temperature on a shaker or rocker platform. The gel was rinsed once for 30 min in phosphate-buffered saline (11) containing 0.02% sodium azide (PBS-A). It was then incubated in 10–20 ml of 1/5 or 1/10 dilution of HeLa MAP antiserum in PBS-A for 6 h or longer to allow formation of antigen-antibody complexes. The unbound antibody was removed from the gel by at least six rinses, 6 h each, in 50 vol PBS-A. The gel was then incubated with fluorescent antiglobulin (rhodamine-labeled goat anti-rabbit IgG, heavy and light chains) (N. L. Cappel Laboratories Inc., Cochranville, Pa.) and diluted 1/20–1/100 in PBS-A for 6 h or longer to allow binding of the second antibody. Unbound fluorescent antibody was removed from the gel by rinsing four times for 6 h in 50 vol PBS-A or until it showed no background fluorescence. Gels were photographed on a shortwave ultraviolet transilluminator (Analtech, Inc., Newark, Del.) using Royal × Pan film (Kodak). Exposure times were 5–15 min and film was developed for 10 min in DK-50 developer (Kodak).

To assist in establishing the identity of Coomassie- and fluorescent-stained bands, the same slab was stained with both Coomassie Brilliant Blue and MAP antiserum. In one procedure the gel was first stained with Coomassie Brilliant Blue, then most of the stain was removed by including activated charcoal in the methanol:acetic acid rinses. Immunofluorescent staining was then carried out as usual. In a second procedure the gel was first stained with MAP antiserum, photographed, and then subsequently stained with Coomassie Brilliant Blue. With both procedures, fluorescent and Coomassie bands coincided.

### Indirect Immunofluorescent Staining of *In Vitro* Microtubules

Drops of polymerized HeLa or brain microtubule solutions were applied to coverslips at concentrations of 1–8 mg/ml and allowed to adsorb for 2 min. Coverslips were rinsed in PBS-A, plunged into methanol at –20°C for 5 min, and air-dried. All operations from this point on were performed at 37°C. After a 5-min rehydration in PBS-A, coverslips were incubated with 20  $\mu$ l of undiluted MAP antiserum or antitubulin IgG for 30 min. Coverslips were rinsed in PBS-A at least three times for 2 h (preferably overnight) and incubated with fluorescein-labeled goat anti-rabbit IgG, heavy and light chains (N. L. Cappel Laboratories Inc.) for 30 min. Coverslips were rinsed in PBS-A at least three times for 2 h and viewed in a Zeiss Universal microscope equipped for epifluorescence with a × 63 phase, 1.4 NA planapochromat objective, and a mercury illuminator. Photographs were taken with Tri-X film (Kodak) using exposure times of 20–45 s for immune serum or IgG staining, and up to 3 min for recording dim images such as preabsorption controls or preimmune serum staining. Printing of photographic negatives employed virtually identical conditions for all negatives.

### Indirect Immunofluorescent Staining of HeLa Cells

HeLa cells grown under conditions described previously (2) were plated onto coverslips 18–96 h before use. Cells were fixed by immersing coverslips in methanol at –20°C for 5 min and air drying. Antibody staining and photography were performed as described above. In some instances phase and fluorescent micrographs were taken in parallel on the same fields.

## Preabsorption of Rabbit Antisera

Because the antisera we obtained did not bind well to native protein, antigen was first precipitated with methanol, then rehydrated in PBS-A. Antisera or antitubulin IgG was preabsorbed by mixing 20  $\mu$ l of antiserum or IgG with 80  $\mu$ l of 8.2 mg/ml HeLa microtubule protein or 8.0 mg/ml HeLa tubulin (purified by DEAE-Sephadex chromatography as described elsewhere (2) and incubating the mixture for 30 min at 37°C. Antisera were also mixed with buffer (80  $\mu$ l PBS-A to 20  $\mu$ l antisera) as a control, but this had no effect on the staining patterns observed.

## RESULTS

### Characterization of HeLa MAP Antisera by Indirect Immunofluorescence of Polyacrylamide Gels

The HeLa MAP protein used in preparing the immunogen for injecting rabbits was obtained from Coomassie Brilliant Blue-stained polyacrylamide gels of purified HeLa microtubule protein. The boxes in Fig. 1 denote the positions of the razor cuts used to excise the material. The 210k group was centered at 210,000 mol wt and consisted of three protein species with electrophoretic mobilities corresponding to 220,000; 208,000; and 200,000 mol wt. The 125k MAP group was named for its major component of 125,000 mol wt. The two major groups of

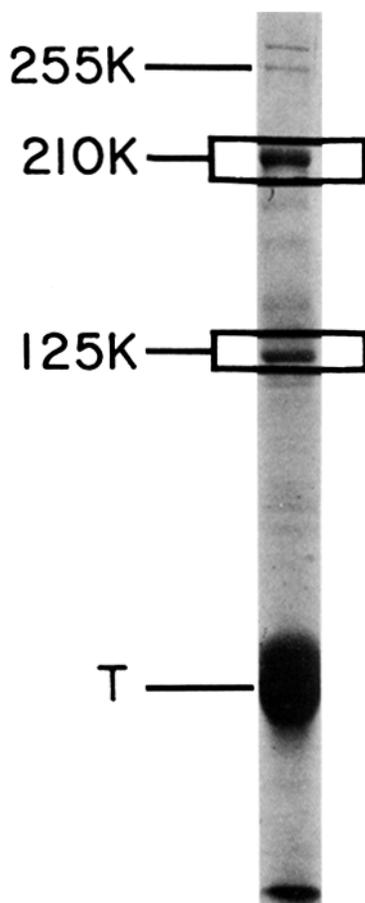


FIGURE 1 Immunogen used to prepare antisera against 210k and 125k HeLa MAPs. HeLa microtubule protein, H<sub>3</sub>P, was electrophoresed on 7.5% acrylamide gels prepared according to Laemmli (14). Black boxes delineate the positions of razor cuts used to excise 210k and 125k MAP regions from the gel for injection into rabbits. Lines at left indicate the positions of the 255k HeLa MAP and tubulin.

MAPs were defined as above because of preliminary evidence that suggested that the species in each group behaved equivalently in a microtubule-binding assay (reference 2 and footnote 1). Each MAP group was injected independently into different rabbits to raise the respective antisera.

The 210k and 125k MAP antisera obtained were assayed for their specificity using an immunofluorescent staining technique for polyacrylamide gels. This procedure has been described in detail in Materials and Methods. Briefly, it consists of electrophoresing proteins in an SDS polyacrylamide slab gel, removing the SDS, and incubating the gel with antiserum and fluorescent antiglobulin. In this way, species in the gel that react with the antibodies can be visualized or photographed with ultraviolet illumination. As we demonstrate below, the assay is sensitive and can be used to measure the specificity of a given antiserum.

The specificity of the MAP antisera is documented in Fig. 2. The figure shows electropherograms of purified HeLa microtubule protein and HeLa extract stained with Coomassie Brilliant Blue (*a* and *b*), 210k MAP antiserum (*c* and *d*), 125k MAP antiserum (*e* and *f*), and preimmune serum from a rabbit subsequently used to produce 125k MAP antisera (*g* and *h*).

The 210k MAP antisera clearly stained proteins of 210,000 mol wt in both the purified microtubule protein and the extract. In addition, the 210k MAP antisera stained a minor species of 255,000 mol wt which was not part of the original immunogen (see Fig. 1). This cross-reacting component present in microtubule protein but not detectable in the extract was previously shown to be an MAP (reference 2 and footnote 1). Preimmune sera from rabbits used to prepare the 210k MAP antisera failed to stain either purified microtubule protein or extract (data not shown).

The specificity of the 125k MAP antisera was also tested (Fig. 2*e-h*). Fluorescence was detected only at the position of the 125k MAP group in both microtubule protein and extracts and no fluorescence was detected when the same samples were stained with preimmune sera from rabbits subsequently injected with 125k MAP. It should be noted that the immunofluorescent staining permitted visualization of components in the crude extracts that were not detectable as distinct bands in Coomassie-stained gels.

To determine the sensitivity of our immunofluorescent gel staining techniques, the ability to detect small amounts of 210k HeLa MAP in gels was tested. Fig. 3 compares the Coomassie Brilliant Blue-staining technique with the immunofluorescent-staining technique. Various amounts of HeLa microtubule protein were electrophoresed on identical slab gels. One gel, shown in Fig. 3 (*a-f*) was stained with Coomassie Blue. The amount of protein in the MAP and tubulin bands of each lane was determined by Lowry protein assays (16) and gel densitometry, as we describe in another report.<sup>1</sup> The smallest amount of 210k MAP detectable in the photograph is the 88 ng load in lane *g*, although smaller amounts could be seen in the stained gel.

The increased sensitivity of the immunofluorescent staining technique is documented in Fig. 3 (lanes *a'-j'*). Using this technique, as little as 8.5 ng of 210k MAP was detected (lane *j'*). The immunofluorescent-staining technique can, therefore, be used under our conditions to detect <10-ng amounts of the 210k MAP in cell extracts or preparations of microtubule protein.

Also included in Fig. 3 (lane *a*) is the Coomassie-stained electrophoretic pattern of a MAP fraction prepared from por-

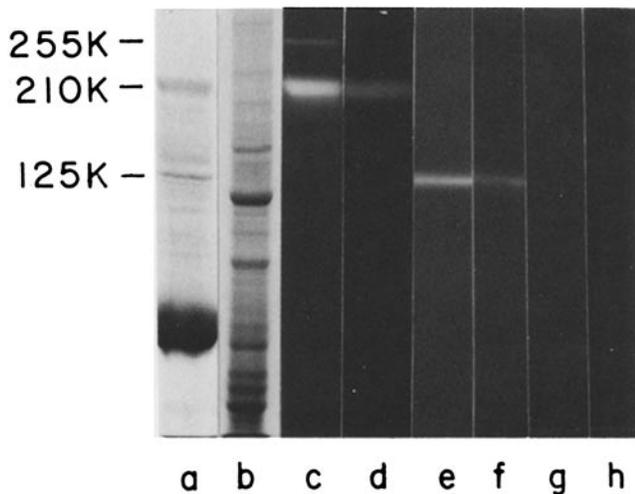


FIGURE 2 Indirect immunofluorescent staining of gels. SDS polyacrylamide gels (6.25% acrylamide) of HeLa microtubule protein (30  $\mu$ g) and of HeLa extract (40  $\mu$ g) were stained by the conventional Coomassie Brilliant Blue staining procedure as shown in lanes a and b, respectively. Identical lanes were stained using the indirect immunofluorescence gel staining procedure with 210k MAP antiserum (c and d), with 125k MAP antiserum (e and f), and with preimmune serum from a rabbit later injected with 125k MAP (g and h). Lines at left indicate the positions of the 255k, 210k, and 125k MAPs.

cine brain microtubule protein. This MAP fraction contains ~2,000 ng of the high molecular weight brain MAP, HMW (also referred to as MAP<sub>2</sub> [28]). It also contains at least 100 ng of each of the minor brain MAPs, MAP<sub>1</sub> of 345,000 mol wt, and the tau proteins, four bands of 55,000–62,000 mol wt. A comparison of lanes a and a' in Fig. 3 demonstrates that there was no detectable cross-reactivity between the HeLa antiserum and porcine brain MAPs. Therefore, the reaction of the HeLa 210k MAP antisera is at least 200-fold weaker with brain HMW MAP than with the homologous 210k MAP. A further test for cross-reactivity of HeLa 210k MAP and brain HMW was to attempt to stain HeLa microtubule protein with antiserum prepared against brain HMW. No cross-reactivity between the HMW MAPs from the two sources was observed with this experimental design.<sup>2</sup>

#### Characterization of HeLa MAP Antisera by Indirect Immunofluorescence of Microtubules Polymerized In Vitro

The antisera were further characterized by staining microtubules polymerized in vitro. In Fig. 4 panels a, d, g, and j show staining results with 210k MAP antisera, panels b, e, h, and k with 125k MAP antisera, and panels c, f, i, and l with antitubulin IgG. Both HeLa MAP antisera (a and b) stained microtubules formed from HeLa microtubule protein as did antitubulin (c). However, neither HeLa MAP antisera stained microtubules polymerized from pig brain microtubule protein (d and e), whereas antitubulin did (f). Thus, the lack of cross-reactivity of the HeLa antisera with brain MAPs evidenced by the gel patterns (Fig. 3) is confirmed by the microscope analysis of in vitro tubules. Several control experiments demonstrate the immunological specificity of the staining reaction. No fluorescent staining was observed when any preimmune serum

was tested against the HeLa microtubules (data not shown). In addition, the preabsorption of 210k (g) or 125k (h) antiserum with purified HeLa tubulin (lacking MAPs) had no effect on staining, while preabsorption with HeLa microtubule protein (containing a full complement of MAPs) prevented staining (j and k). When antitubulin was preabsorbed with either HeLa microtubule protein (i) or purified HeLa tubulin (l) no staining of HeLa microtubules was observed. Thus, anti-MAP staining of HeLa microtubules polymerized in vitro demonstrates that the 210k and 125k MAPs are specifically associated with the tubules and are distributed along their entire length.

#### Characterization of the HeLa MAP Antisera by Indirect Immunofluorescence of Cells

Indirect immunofluorescent staining was used to examine the physical association of the 210k and 125k MAPs with microtubules in vivo. In interphase cells, antisera to the 210k MAP stained a network of cytoplasmic fibers that closely resembled the network stained with antitubulin immunoglobulin (compare panels a and d, Fig. 5). Fibers focused on an area near the nucleus, which probably contains the microtubule-organizing center. In mitotic cells, the spindle stained with 210k MAP antisera, as demonstrated by panels b and c in Fig. 5. The metaphase spindle in panel b is very similar to the spindle stained with antitubulin in panel e.

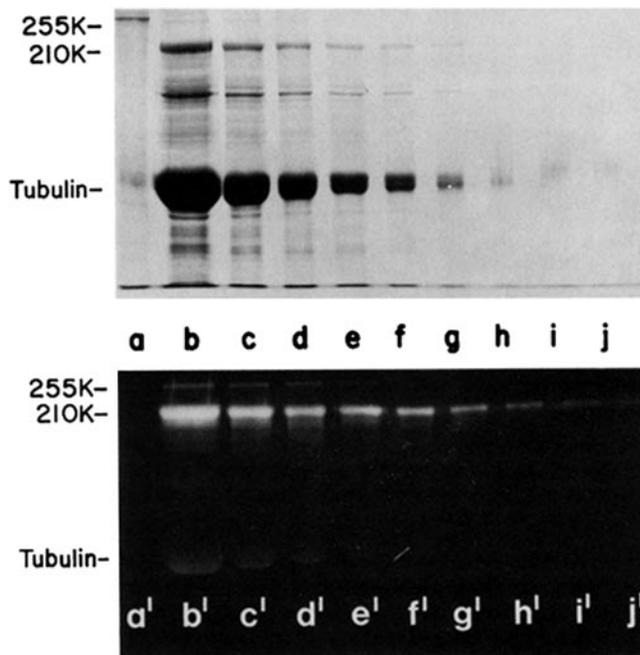


FIGURE 3 Sensitivity of immunofluorescence gel assay for detection of 210k MAP. Samples of brain MAPs and HeLa microtubule protein were electrophoresed on two identical 7.5% acrylamide slab gels and were stained with Coomassie Brilliant Blue (lanes a-j) or indirect immunofluorescence (lanes a'-j'). Gel lanes a and a' contained 20  $\mu$ g of a brain DEAE MAP fraction prepared by John G. Peloquin according to the procedure of Vallee and Borisy (30). Lanes b, b'-j, j' contained the following amounts of HeLa microtubule protein, C<sub>3</sub>S, corresponding to the following amounts of 210k MAP. b, b': 140  $\mu$ g C<sub>3</sub>S, 3,500 ng 210k MAP; c, c': 56  $\mu$ g C<sub>3</sub>S, 1,400 ng 210k MAP; d, d': 28  $\mu$ g C<sub>3</sub>S, 700 ng 210k MAP; e, e': 14  $\mu$ g C<sub>3</sub>S, 350 ng 210k MAP; f, f': 7  $\mu$ g C<sub>2</sub>S, 175 ng 210k MAP; g, g': 3.5  $\mu$ g C<sub>3</sub>S, 88 ng 210k MAP; h, h': 1.4  $\mu$ g C<sub>3</sub>S, 35 ng 210k MAP; i, i': 0.7  $\mu$ g C<sub>3</sub>S, 17 ng 210k MAP; and j, j': 0.35  $\mu$ g C<sub>3</sub>S, 8.5 ng 210k MAP. Lines at left indicate the positions of 255k MAP, 210k MAP, and tubulin in the electropherograms.

<sup>2</sup> Peloquin, J. G. Unpublished results.

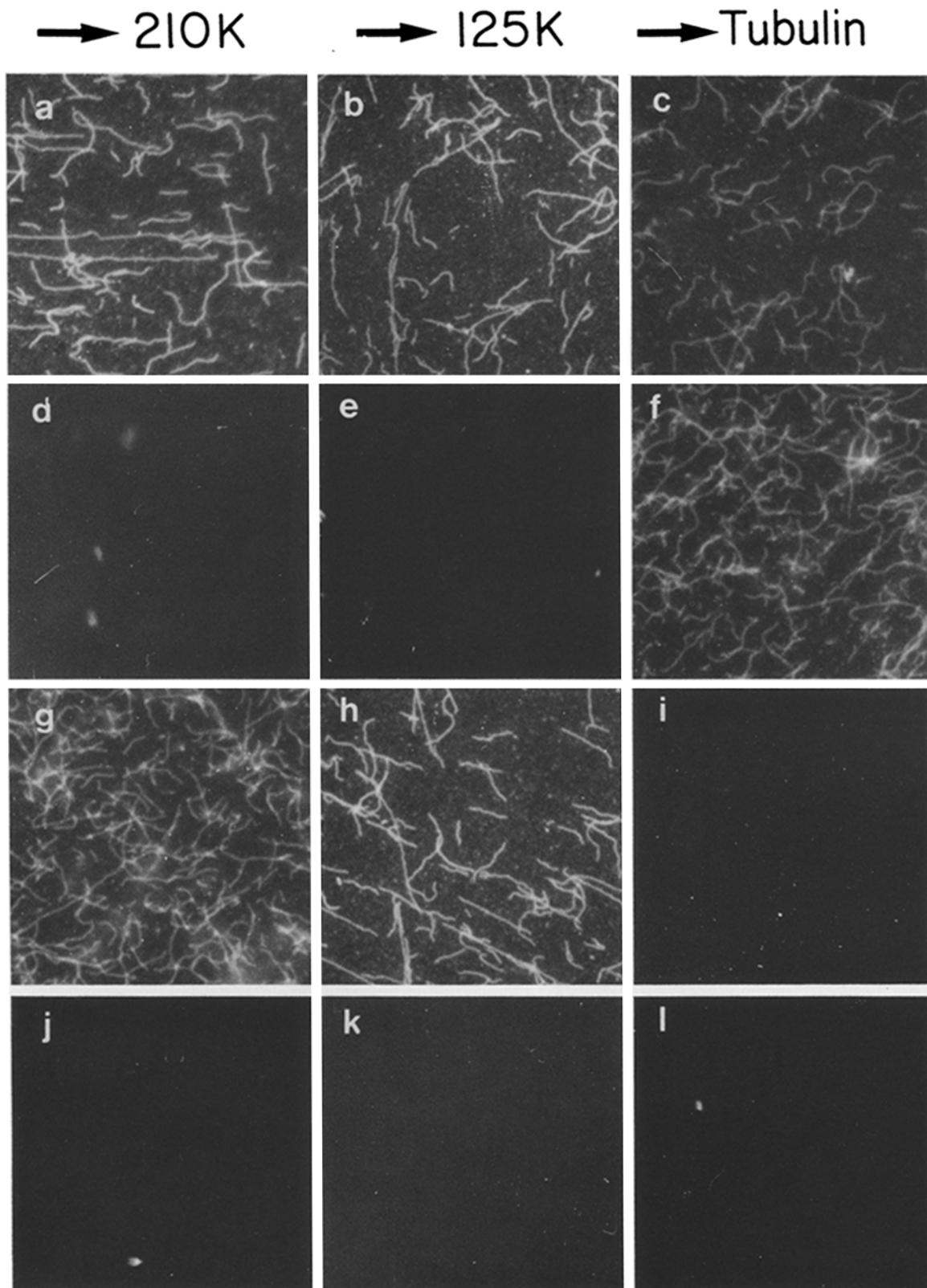


FIGURE 4 Indirect immunofluorescent staining of *in vitro* polymerized microtubules. Preparations of microtubule protein were polymerized, applied to coverslips, and stained with HeLa MAP antisera or antitubulin IgG as described in Materials and Methods. Panels a-c, HeLa microtubules stained with 210k, 125k antisera and antitubulin IgG, respectively. Exposure times were 20, 30, and 45 s. Panels d-f, porcine brain microtubules stained with 210k, 125k, and antitubulin. Exposure times were 3 min for d and e, 20 s for f. Panels g-i, staining of HeLa microtubules with 210k, 125k MAP antisera, and antitubulin IgG preabsorbed with DEAE-purified HeLa tubulin (see Materials and Methods). Exposure times were 35 s for g and h, 35 min for i. Panels j-l, staining of HeLa microtubules with 210k, 125k MAP antisera and antitubulin IgG preabsorbed with HeLa microtubule protein containing a full complement of HeLa MAPs. Exposure times were 2.5 min for j and 3 min for k and l.  $\times 1,500$ .

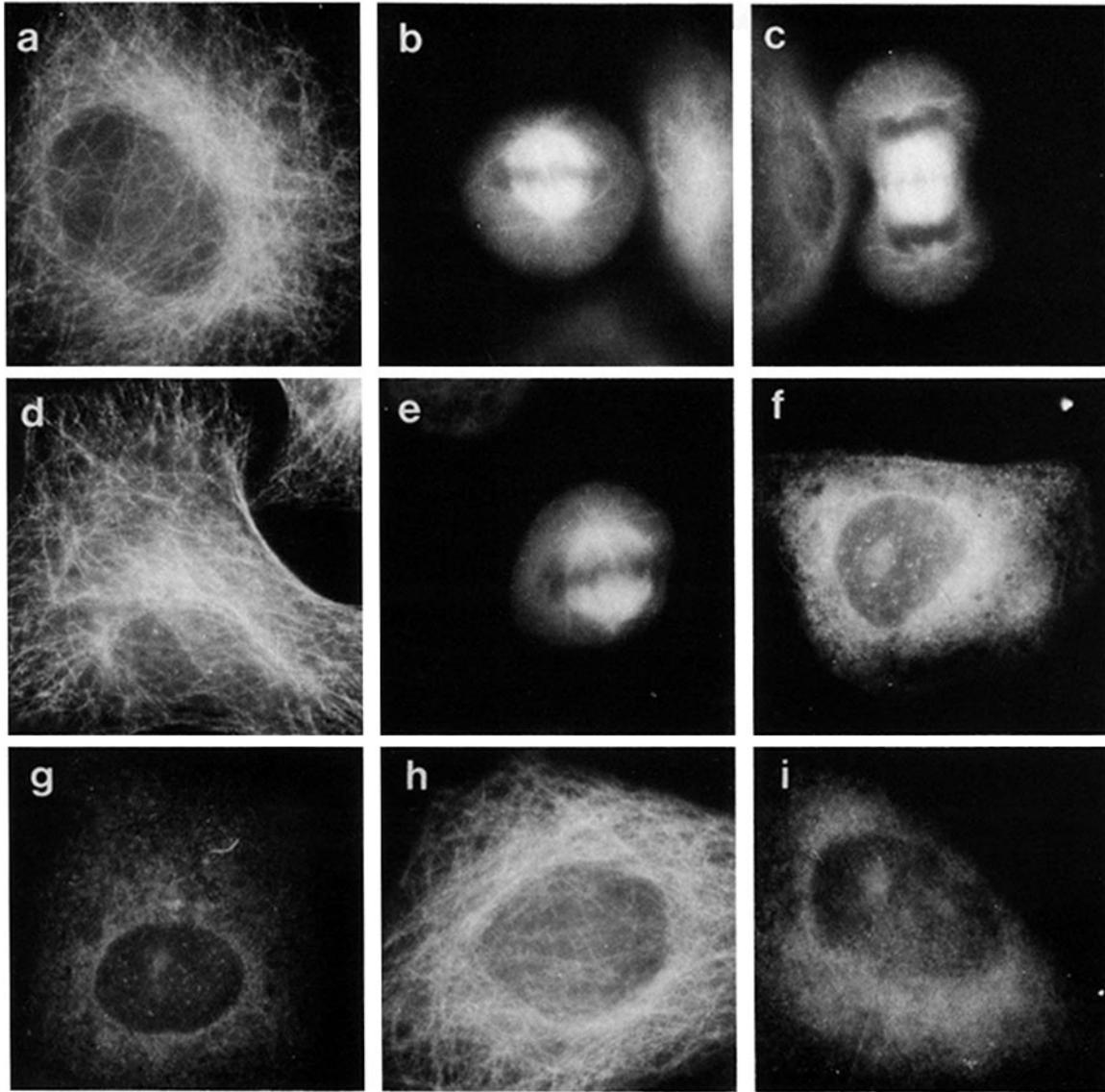


FIGURE 5 Immunofluorescent staining of fixed HeLa cells with antisera against 210k MAP. (a) Interphase cell stained with 210k MAP antiserum. Exposure time, 35 s.  $\times$  950. (b) Metaphase cell, 210k MAP antiserum. Exposure time, 15 s.  $\times$  700. (c) Anaphase cell, 210k MAP antiserum. Exposure time, 25 s.  $\times$  700. (d) Interphase cell stained with antitubulin IgG. Exposure time, 25 s.  $\times$  830. (e) Metaphase figure, antitubulin. Exposure time, 12 s.  $\times$  750. (f) Anti-210k-stained interphase cell that was pretreated with 0.06  $\mu$ g/ml Colcemid for 2 h. Exposure time, 40 s.  $\times$  750. (g) Interphase cell stained with preimmune serum from a rabbit subsequently injected with 210k MAP. Exposure time, 3.5 min.  $\times$  750. (h) Interphase cell stained with 210k MAP antiserum preabsorbed with HeLa tubulin. Exposure time, 40 s.  $\times$  950. (i) Interphase cell stained with 210k MAP antiserum preabsorbed with HeLa microtubule protein containing a full complement of HeLa MAPs. Exposure time, 3.5 min.  $\times$  950.

The staining observed along cytoplasmic fibers and in mitotic spindles was probably localized on microtubules, as treatment of cells with 0.06  $\mu$ g/ml Colcemid for 2 h disrupted the staining pattern (Fig. 5, panel *f*). The cells that were Colcemid-treated stained with approximately the same intensity as untreated cells but the staining was diffuse or granular instead of fibrous.

Three control experiments demonstrated that the staining was caused by an immunochemical reaction with MAP molecules in the cytoskeleton. Panel *g* contains a cell stained with preimmune serum. Staining was much dimmer than in the cells stained with 210k MAP antiserum; the exposure time for panel *g* was six times longer than for panel *a*. No organized pattern of stain was seen and the diffuse fluorescence was brightest near the nucleus. Panel *h* shows a cell stained with 210k MAP antiserum which was preabsorbed with ion-exchange purified HeLa tubulin, without MAPs. The fibrous staining pattern was

equivalent to the pattern obtained with untreated serum and the intensity of staining was similar. When cells were stained with 210k MAP antisera which was preabsorbed with unfractionated HeLa microtubule protein (which contains HeLa MAPs), the result was equivalent to that with preimmune serum (compare panels *g* and *i*). Again, only dim, diffuse staining was observed and photographs were made with longer exposure times.

In some cases, we recorded cells in parallel phase and immunofluorescence images. Fig. 6 contains three such pairs of dividing cells stained with 210k MAP antisera. Panels *a* and *b* show a tripolar cell (not uncommon in HeLa) during prophase. Note the slender processes in the phase micrograph (*a*) which hold the rounded cell to the substrate. In the immunofluorescence micrograph (*b*) many fibers originate at each of the three poles. Even in this round cell it is easy to see the

microtubules which fill the extranuclear space.

In metaphase (panels *c* and *d*), staining was observed throughout the spindle but was excluded from the area where the chromosomes lie. Note again the slender connections to the substrate.

Panels *e* and *f* show a pair of cells joined by a midbody. The

central phase-dense region that does not stain is flanked by staining regions of brightness corresponding to density in the phase image. The two daughter cells have spread almost completely by this stage and there are abundant cytoplasmic microtubules once again.

In cells treated with 125k MAP antiserum, the patterns

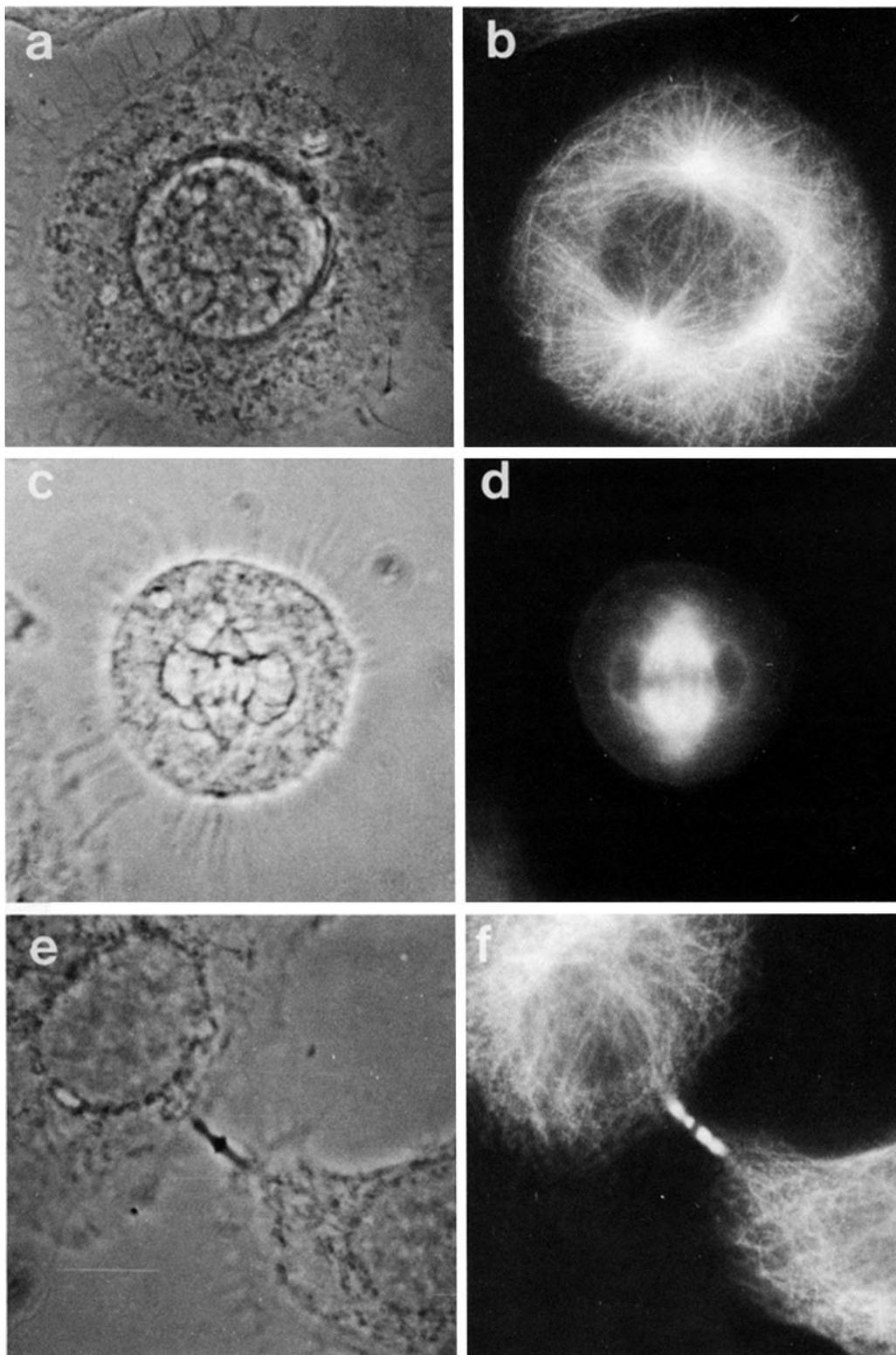


FIGURE 6 Phase and immunofluorescence pairs of dividing HeLa cells stained with 210k MAP antisera. (a and b) Prophase.  $\times$  1,400. (c and d) Metaphase.  $\times$  1,000. (e and f) Midbody.  $\times$  1,000.

obtained were indistinguishable from those obtained with 210k MAP or tubulin antibodies. Fig. 7 shows an interphase cell (panel *a*), a metaphase phase and immunofluorescence pair (*b* and *c*), another metaphase (*d*), and a telophase example (*e*). These are comparable to the examples included in Figs. 5 and 6 in which cells were stained with anti-210k or antitubulin antibodies. Note that the nature of the interphase cell staining, as shown in panel *a*, resembles anti-210k MAP or antitubulin staining. The metaphase examples show typical patterns, and panel *e* looks like panel *f* of Fig. 6.

As with 210k MAP antisera, the staining with 125k antiserum was Colcemid-sensitive (Fig. 7*f*) and the pattern was not caused by antibodies in the preimmune serum (Fig. 7*g*). A preabsorption experiment like the one performed for 210k showed that the staining could be preabsorbed by MAP molecules and not by purified tubulin (panels *h* and *i*). Therefore,

the 125k as well as the 210k MAP appear to be distributed along the length of microtubules in the cytoplasmic fibers, mitotic spindles, and midbodies of fixed HeLa cells. Although we have observed anti-MAP staining in all of the places where microtubules usually are localized and although the immunofluorescent staining patterns of antitubulin and HeLa MAP antibodies look very similar, we cannot be sure that there are not some microtubules or parts of microtubules that are devoid of the two major HeLa MAPs. A test of such a possibility would require a combination of electron microscopy and immunofluorescence on the same cell or immunoelectron microscopy.

## DISCUSSION

In this report, the high degree of specificity of the antisera against 210k and 125k HeLa MAPs was demonstrated using

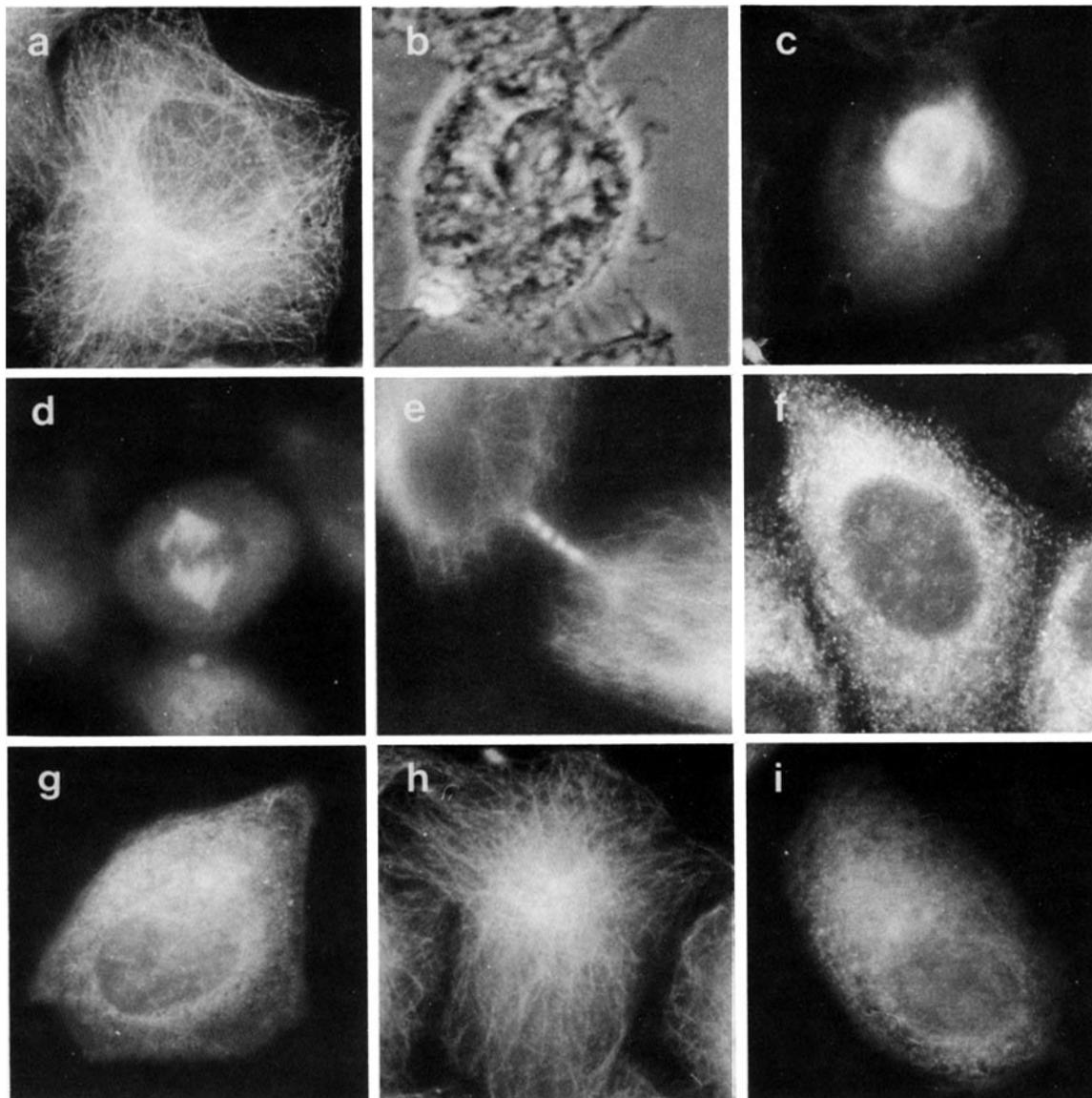


FIGURE 7 Immunofluorescent staining of fixed HeLa cells with antisera against 125k MAP. (*a*) Interphase example, stained with 125k antiserum; 45-s exposure. (*b* and *c*) Phase and immunofluorescence pair of metaphase figures; *c* is a 20-s exposure. (*d* and *e*) Metaphase and telophase cells, stained with 125k MAP antisera; 30-s exposures. (*f*) 125k MAP antibody staining pattern of a cell pretreated with Colcemid; 45-s exposure. (*g*) Interphase cell stained with preimmune serum from a rabbit subsequently injected with 125k MAP; 3-min exposure. (*h*) Interphase cell stained with 125k MAP antiserum preabsorbed with HeLa tubulin; 40-s exposure. (*i*) Interphase cell stained with 125k MAP antiserum preabsorbed with HeLa microtubule protein containing a full complement of HeLa MAPs; 3-min exposure.  $\times 750$ .

an immunofluorescent staining technique for polyacrylamide gels. The method represents a modification of a technique used by Olden and Yamada (20), differing primarily in that fluorescence rather than horseradish peroxidase was used to localize the antigen. The method was sufficiently sensitive to detect <10-ng amounts of antigen in slab gels. Assuming the same detection limit for cell extracts, a gel loading of 100  $\mu$ g would permit the detection of 210k MAP if it comprised  $\geq 0.01\%$  of the cell protein. Based on the amount of HeLa extract that must be electrophoresed to visualize the 210k MAP by the immunofluorescence assay, we estimate that it constitutes  $\sim 0.04 \pm 0.01\%$  (wt/wt) of the soluble protein.

The determination of the amount of HeLa MAP in crude extracts permits us to make estimates, albeit rough and tentative, of the relation between MAP and tubulin in the cell. We have previously established that the proportion of soluble protein in HeLa cells which is tubulin is  $\sim 4\%$  (wt/wt) (3). Therefore, the proportion of 210k MAP to tubulin in the crude extracts is estimated at  $\sim 1\%$  (0.04/4%). This compares to a proportion of 2.5% in microtubule protein prepared from crude HeLa extracts by assembly-disassembly cycling (2). This may signify that the proportion of MAPs bound to microtubules is greater than the proportion of tubulin in microtubules. In another study,<sup>1</sup> we have estimated the native molecular weight of 210k MAP from its solution behavior. Based on a value of 409,000 daltons, 210k MAP would appear to be present in HeLa extracts at a molar ratio of approximately one MAP:400 tubulin dimers. Using the 2.5% figure, the ratio for cycled microtubules would be one MAP:160 tubulin dimers. Assuming a value of 1,625 dimers per micrometer length of microtubule, this proportion corresponds to  $\sim 10$  MAP molecules per micrometer.

In staining gels with MAP antisera, we observed some staining in the tubulin band (Fig. 3, lanes *a'-e'*). We feel that this staining is nonspecific for two reasons: (a) It occurs only in heavily loaded bands ( $\geq 14 \mu$ g, or 1,400 times the detection limit for HeLa 210k MAP). (b) The activity of our antisera to stain microtubules or cells was not removed by preabsorbing with large amounts of tubulin (see, for example, Figs. 4g, 4h, 5h, and 6h).

The antisera prepared against the 210k group of MAPs also reacted with a minor MAP of 255,000 mol wt. The 255k MAP could be a precursor molecule to the 210k MAP, but we have no additional evidence for its role. In contrast to the 210k MAP antisera, the 125k MAP antisera bound only to material of 125,000 mol wt.

It should be noted that neither the 210k antisera nor the 125k antisera reacted with the MAPs (HMW, tau) derived from brain tissue; thus, the brain MAPs and HeLa MAPs appear to be immunologically distinguishable species.

By use of the specific antisera, both the 210k and 125k HeLa MAPs were found to be specifically associated with fixed microtubules *in vitro*. We have demonstrated elsewhere<sup>1</sup> that the HeLa MAPs contribute to initiation and elongation of microtubules. This finding is supported by the observation that fluorescent staining obtained with either MAP antiserum is present along the length of the microtubule.

We have also examined the intracellular distribution of the 210k and 125k MAPs in fixed HeLa cells. Both the 210k and 125k MAPs were found in association with a network of Colcemid-sensitive fibers in the cytoplasm of interphase HeLa cells. In mitotic HeLa cells, 210k and 125k MAPs were localized in the mitotic spindle, and in cells undergoing cytokinesis they were densely packed in the midbody. Because the 255k MAP

appears to cross-react with the 210k MAP antisera, and because all staining is localized on cytoplasmic and spindle microtubules, the 255k MAP may also be specifically localized on microtubules *in vivo*.

The association of putative MAPs with microtubules *in vivo* has previously been questioned. Lee et al. (15) argued that the association and copurification of molecules with tubulin may be caused by electrostatic interactions between the anionic protein, tubulin, and some cationic portion of the putative MAP. Because of this possibility, Lee et al. suggested that no conclusions about the physiological significance of a MAP should be drawn from solution experiments.

Cleveland et al. (7) also pointed out the possibility of non-specific binding of proteins to tubulin in the concentrated cell extracts prepared for the purification of microtubule protein. To avoid this adventitious association, Cleveland et al. have isolated microtubule protein from cultured cells by copolymerization of dilute, highly radioactive extracts with carrier microtubule protein. In copolymerization experiments like theirs (4), we isolated the 210k and 125k MAPs and the results presented in this report demonstrate the *in vivo* association of the MAPs with microtubules. Therefore, nonspecific binding of microtubules to tubulin in extracts is not the source for the 210k and 125k HeLa MAPs.

Solomon et al. (29) have also raised concern about the possible misidentification of contaminants as MAPs. In novel experiments designed to circumvent this problem, Solomon and co-workers carried out cycles of assembly-disassembly on detergent-solubilized cytoskeletons and were able to identify a MAP of 69,000 mol wt. The major limitation of this kind of experiment is that only those MAPs that are extracted from the cytoskeleton under the same conditions as used for tubulin would be isolated in the cytoskeleton preparations. A MAP might associate reversibly with tubulin and dissociate during the extreme dilutions used in extraction of the cell. Preliminary evidence from this laboratory<sup>3</sup> indicates that both the brain and HeLa MAPs would be lost from the cytoskeleton under these extraction conditions. Also, as Solomon et al. point out, a MAP might function in the cell to bind microtubules to other structures or filament systems and it might be extracted from cytoskeletons less easily than tubulin. Hence, the procedure used by Solomon to exclude contaminants may identify a subset of MAPs, possibly distinct from those we have found.

The evidence presented in this report represents a demonstration that molecules that are bound to microtubules polymerized *in vitro* are also bound to microtubules in the cell from which they were extracted. The identification of the 210k and 125k MAPs on microtubules in fixed cells using highly specific, well-characterized antibodies permits speculation about their role in the polymerization or function of microtubules in cultured cells. Because these proteins seem to be present along microtubules in HeLa cells at all stages of the cell cycle, it is unlikely that they are involved in the function of specific types of microtubules. On the other hand, as these molecules significantly lower the critical concentration for microtubule polymerization *in vitro*, they may serve a similar function in cells. HeLa MAPs could also serve enzymatic functions or could serve to cross-bridge microtubules. In any case, the evidence presented here for the presence of HeLa MAPs along microtubules in fixed cells strongly suggests that the 210k and 125k MAPs are physiologically significant components of microtubules in cultured HeLa cells.

<sup>3</sup> Peloquin, J. G., J. C. Bulinski, and G. G. Borisy. Unpublished results.

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