Studies of a Microtubule-associated Protein Using a Monoclonal Antibody Elicited Against Mammalian Mitotic Spindles

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ABSTRACT   We have devised a procedure for the identification of individual molecules which are associated with the mitotic spindle apparatus and cytoskeleton in mammalian cells. We prepared monoclonal antibody–producing hybridomas by immunizing mice with mitotic spindles isolated from cultured HeLa cells. Among several antibody-producing clones obtained, one hybridoma (22MA2) produced an antibody that recognizes a putative microtubule-associated protein which exhibits unusual distribution characteristics in cultured cells. Immunofluorescence studies showed that during mitosis the 22MA2 antigen is distributed in parallel with the spindle fibers of the mitotic apparatus, and that during interphase the antigen is always associated to a limited extent with cytoplasmic microtubules. Also, the co-distribution of the antigen with microtubules was found to be Colcemid sensitive. However, the 22MA2 antibody immunofluorescently stained the nuclei of cells in the exponential growth phase, but did not stain the nuclei of cells that had grown to confluence. This nuclear fluorescence appears to be directly related to cell density rather than nutritional (serum) factors in the growth medium. The results suggest that the antigen undergoes some change in structure or distribution in response to changes in the proliferative capacity of the cell. Biochemical analyses of cytoplasmic, nuclear, and mitotic spindle subcellular fractions show that the antigen exhibits a polypeptide molecular weight of 240,000, is found in various mammalian cells ranging from marsupial to human, and is particularly susceptible to proteolysis.

Microtubules constitute a major portion of the interphase cytoskeleton and the mitotic spindle fibers in eucaryotic cells. It is now commonly recognized that microtubule protein subunits (tubulin) can be induced in vitro to undergo polymerization and depolymerization in response to a variety of stimuli. However, the endogenous factors that control microtubule assembly and disassembly in vivo remain largely unknown. Much of the recent work in this field has been directed toward elucidating the cellular factors that control microtubule functions.

Evidence has accumulated that proteins other than tubulin are responsible for the cellular control of microtubule assembly and disassembly. Workers have identified several microtubule-associated proteins (MAPs)[1] by taking advantage of the fact that some proteins are found associated with microtubules purified from cell extracts by cycles of assembly and disassembly. Using this approach, investigators have found MAPs in isolates from brain tissue (8, 20, 22, 36) as well as from cultured cells (4, 7, 23, 24, 33, 37).

Because brain is composed largely of differentiated tissue, much work has recently been focused on the MAP composition of proliferating cultured cells. The most extensive catalogue of non-neural MAPs to date has been made using microtubule proteins isolated by the assembly/disassembly method from cultured HeLa cell extracts. Studies have shown that this type of preparation, when analyzed using SDS PAGE, contains MAPs with molecular weights grouped in the ranges of 200,000–220,000 and 97,000–125,000 (4, 33), with some

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[1] *Abbreviations used in this paper:*   BHK-21, baby hamster kidney; DME, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; MAP, microtubule-associated protein; MEM, Eagle's minimal essential medium; PMSF, phenylmethylsulfonyl fluoride.

minor components detected at ~250,000 (4, 33) and 68,000 (33). Immunocytochemical studies on cultured cells have shown that several of these HeLa MAP species are associated with the general cellular microtubule population (5, 13), while another study presented evidence that one MAP species of the 200,000-mol-wt cluster is specifically associated with the mitotic apparatus and the interphase nucleus (12).

The aforementioned studies have approached the identification of MAPs by the initial selection for those proteins that co-assemble with microtubules in vitro. While this is clearly a technically useful criterion, other investigators have pointed out that co-assembly in vitro is not an absolute prerequisite for determining whether or not a protein can be considered a MAP in vivo (17, 30, 33). Also, the in vitro section for MAPs that co-assemble with microtubules could exclude proteins that may nevertheless be integral to microtubule functions in the cell.

Our interest in identifying and characterizing nontubulin components of the mammalian mitotic spindle has led us to a slightly different approach to the problem. It is difficult to isolate mitotic spindles from cultured cells (or any other mammalian cells) in sufficiently pure quantities for biochemical analyses of discrete protein components. However, we reasoned that if enough spindles could be isolated for use as crude antigens to elicit an immunological response in mice, hybridomas could be produced that might secrete monoclonal antibodies against nontubulin components of the mitotic apparatus. In this report we describe the production of such an antibody, as well as its use in the initial characterization of an unusual microtubule-associated protein.

MATERIALS AND METHODS

Cell Culture: The cell lines PtK₂ (rat kangaroo kidney epithelium), BRL-3A (rat liver epithelium), 3T3 (mouse embryonic fibroblast), BHK-21 (hamster kidney fibroblast), R9ab (rabbit lung fibroblast), Chinese hamster ovary, HeLa (human cervical carcinoma), and WI-38 (human lung fibroblast) were all obtained from the American Type Culture Collection, Rockville, MD. The mouse plasmacytoma (myeloma) line NS-1 was originally obtained from the Salk Institute Cell Distribution Center, San Diego, CA. PtK₂, BRL-3A, BHK-21, R9ab, Chinese hamster ovary, HeLa, and WI-38 cultures were grown in Eagle's minimal essential medium (MEM) supplemented with Earle's salts and 10% fetal calf serum (FCS). 3T3 and NS-1 cultures were grown in Dulbecco's modified Eagle's medium (DME) containing 10% FCS. Established hybridoma clones (see below) were grown in DME containing 10% HyClone FCS (Sterile Systems, Inc., Logan, UT). All cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 (for cultures in MEM) or 7.5% CO_2 (for cultures in DME).

Mitotic Spindle Isolation: HeLa cells were grown (as monolayers) to 50% confluence in large T-flasks, and were partially synchronized by a single excess thymidine block (2). After removal of the thymidine blockade, cells were allowed to progress into mitosis where they were arrested by the addition of 1.3 × 10⁻⁷ M nocodazole to the growth medium (38). Mitotic cells were allowed to accumulate for 3–5 h in the presence of nocodazole before they were harvested by vigorously shaking the flasks. The cells were then pelleted by centrifugation, washed once with fresh medium, and resuspended in drug-free medium at 37°C. More than 90% of the cells formed metaphase spindles (as determined by phase-contrast microscopy) after a 30-min recovery from the effects of the nocodazole.

Mitotic spindles were isolated from the synchronized mitotic cells using modifications of the method of Chu and Sisken (6), and all procedures were performed at 37°C. After removal of the medium by centrifugation, the cells were washed twice with PBS. Cell pellets were then resuspended in 50 vol of swelling buffer (1 mM sodium phosphate, 1 M hexylene glycol, pH 6.5) and centrifuged at 500 g for 3 min. Swelling buffer was removed, the pellets of swollen cells were resuspended in 10–20 vol of lysing buffer (1 mM sodium phosphate, 1 M hexylene glycol, 0.25% Triton X-100, 1 mM EGTA, pH 6.5), and the cells were disrupted by vigorous vortexing for ~20 s. The lysates were monitored by phase-contrast microscopy for the presence of intact metaphase spindles free of cytoplasmic debris. Preparations were further used only if they

contained ≥95% spindles (with the remaining ≤5% consisting of unbroken cells and/or nuclei). We quantitated spindles by examining lysate samples in a hemacytometer with a phase-contrast microscope. Lysates were then chilled to 4°C and the mitotic spindles were pelleted by centrifugation at 800 g for 10 min. After removal of the supernatant, spindles were washed once with lysing buffer and either stored as frozen pellets or processed for electrophoretic analysis (see below).

Monoclonal Antibody Production: BALB/c or CB6F₁/j mice were immunized intraperitoneally with 1–2 × 10⁷ HeLa spindles emulsified in Freund's complete adjuvant. The mice were boosted 2–4 wk later with the same number of spindles emulsified in Freund's incomplete adjuvant. 4 d later the animals were killed, spleens were removed and dissociated, and the spleenocytes were fused with NS-1 myeloma cells (at a 5:1 ratio of spleenocytes/myeloma cells) according to the general procedures of Kohler and Milstein (15) as modified by Kennett (14). Fused cells were resuspended in hypoxanthine/aminopterin/thymidine-selective DME (19) and distributed into four 24-well culture plates for each spleen equivalent of fused cells.

When hybridoma cultures achieved 60% confluence, their culture supernatants were assayed by immunofluorescence as follows. PtK₂ cells were inoculated into 24-well culture plates containing a sterile round 6-mm coverslip in each well, and were allowed to grow to 60–70% confluence. Growth medium was removed and coverslip cultures were fixed with 3.7% formaldehyde in PBS, followed by three washes with PBS containing 0.05% Triton X-100. Individual wells containing fixed coverslip cultures then received samples of growth medium from individual hybridoma culture wells described above. After incubation with hybridoma supernatants for 2 h at 37°C, the coverslip cultures were washed three times with PBS (5 min each) and then incubated with a 1:100 dilution (in PBS) of fluorescein-conjugated goat anti-mouse Igs (Antibodies, Inc., Davis, CA) at 37°C for 30 min. Coverslips were then washed three times with PBS, removed from the assay wells, and mounted with Aquamount (Lerner Labs, New Haven, CT). Coverslips were examined by fluorescence microscopy for mitotic cells that showed positively stained mitotic spindles. Hybridomas found by the assay to secrete the desired antibodies were harvested and cloned at least twice by limiting dilution. The hybridoma clones were then grown up in flasks, and their culture supernatants were harvested and used as the source of monoclonal antibodies for subsequent experiments.

General Immunofluorescence Procedures: Cells were grown as monolayers on sterile 11 × 22 mm coverslips in 60-mm culture dishes. Coverslip cultures were briefly rinsed with PBS and usually fixed with 3.7% formaldehyde in PBS for 20 min at 23°C. In some experiments, fixation methods using glutaraldehyde (35) or cold methanol (25) were used for comparison with the formaldehyde procedure. The cells were then washed once with PBS containing 0.05% Triton X-100 and twice with PBS alone, followed by a 1-h incubation with the appropriate primary antibody at 37°C. Primary antibodies included hybridoma supernatants containing mouse monoclonal antibody (described above, undiluted) or rabbit antibody directed against tubulin (prepared by the method of Fuller et al. [10]) diluted 1:20 in PBS. Controls included preimmune mouse and rabbit sera diluted in PBS. Cells were then freed of excess primary antibody by three washes with PBS and incubated with the appropriate fluorescently labeled secondary antibody for 30 min at 37°C. Secondary antibodies were diluted 1:100 in PBS and included fluorescein-conjugated goat anti-mouse IgG + IgM (Boehringer Mannheim Biochemicals, Indianapolis, IN) or rhodamine-conjugated goat anti-rabbit IgG (U. S. Biochemical Corp., Cleveland, OH). Following fluorescent labeling, coverslips were washed three times with PBS, rinsed with distilled water, and mounted with Aquamount for observation. Coverslips were examined with a Zeiss epifluorescence microscope equipped with the appropriate excitation filters (Zeiss filter set No. 487709 for fluorescein and set No. 487714 for rhodamine). Fluorescent images were recorded on Kodak Tri-X Pan film using 1-min exposures for all photomicrographs.

Preparation of Cell Extracts and Subcellular Fractions: Cells exponentially growing in 150-mm tissue culture dishes were harvested with a rubber policeman and washed three times with PBS. All subsequent operations were carried out at 4°C unless otherwise noted. For whole-cell extracts, cell pellets were resuspended directly in electrophoresis sample buffer (16). For cytoplasmic and nuclear fractions, cells were resuspended in PBS containing 0.25% Triton X-100 and disrupted with two to three strokes in a Dounce homogenizer, and the nuclei were pelleted by centrifugation. The resulting supernatant (cytoplasmic fraction) was removed and combined with an equal volume of 2X sample buffer. The pelleted nuclei were either stored frozen or resuspended in sample buffer. The effect of protease inhibitors was tested by using one of the following compounds (all obtained from Sigma Chemical Co., St. Louis, MO) in all extraction buffers: aprotinin (100 KIU/ml); phenylmethylsulfonyl fluoride (PMSF, 0.1 mM); pepstatin A (10 μM); or leupeptin (10 μM). In some experiments the isolations were carried out at 23°C with or without protease inhibitors in the extraction buffers, and the isolates were either stored under various conditions or were used immediately in electrophoretic

analyses. Protein concentrations were estimated using the Schacterle and Pollack (28) modification of the method of Lowry et al. (21).

Electrophoretic Procedures: Before electrophoresis, all extracts were sonicated in sample buffer containing 0.0625 M Tris-HCl, 2% SDS, 5 mM dithiothreitol, 1 mM EDTA, 10% glycerol, and 0.001% bromophenol blue as tracking dye. Samples were then boiled for 2 min and analyzed by SDS PAGE (16) using 0.75-mm thick slab gels. Prestained molecular weight standards (Bethesda Research Labs, Gaithersburg, MD) were run in every gel and included myosin heavy chain (200,000 mol wt), phosphorylase b (92,500), BSA (68,000), and ovalbumin (43,000). Additional molecular weight standards included MAP-1 (340,000 mol wt), MAP-2 (280,000), and tubulin (55,000). Duplicate sets of samples were electrophoresed in each gel. Following electrophoresis each gel was cut in half and one half was fixed, stained, and destained with Coomassie Blue according to Fairbanks et al. (9). The other half was subjected to electrophoretic transfer of the protein patterns (Western blotting) onto nitrocellulose membrane using the method of Towbin et al. (31), except that the nitrocellulose sheet was sandwiched on the anodal side of the gel. Transfer efficiencies were determined by Coomassie Blue-staining the transferred gels and densitometrically comparing them with the duplicate samples run in the nontransferred halves of the gels. Transfer efficiencies were typically ≥93% for proteins below 170,000 mol wt and ≥65% for those above that molecular weight. It was also found that the prestained molecular weight standards efficiently transferred onto the nitrocellulose, which greatly simplified calibrations of the Western blots.

Antibody Staining of Western Blots: All procedures were carried out at 37°C unless otherwise noted. After electrophoretic transfer (see above), Western blots were incubated for 30 min in PBS containing 3% BSA to block remaining protein binding sites on the nitrocellulose. Blots were then incubated with the following sequence of reagents for 30 min each: (a) hybridoma supernatant (containing mouse monoclonal antibody) diluted 1:1 with 3% BSA in PBS; (b) biotinylated horse anti-mouse Igs (Vector Labs, Burlingame, CA) diluted 1:200 with 1.5% BSA in PBS; and (c) peroxidase-conjugated avidin (Vector Labs) diluted 1:100 with 1.5% BSA in PBS. All reagent incubations were carried out by overlaying each Western blot with a sheet of Whatman No. 1 filter paper cut to size and saturated with reagent at 25 μl/cm^2 of filter paper. After each reagent incubation the blots were washed twice at 23°C with PBS containing 0.05% Triton X-100 and then twice with PBS alone. To visualize the sites of peroxidase antibody bound to antigen, we incubated the blots 7 min at 23°C in PBS containing 0.005% 4-chloro-1-naphthol and 0.001% H$_2$O$_2$. The blots were rinsed with distilled water after color development and were air-dried.

RESULTS

HeLa mitotic apparatuses were obtained by disrupting synchronized metaphase cells in a buffer that stabilizes the structure of mitotic spindles (see Materials and Methods and reference 6). As reported by other investigators, the isolated spindles are cold stable and free of chromosomes (presumably owing to the effects of the hexylene glycol and the hypotonic nature of the buffer, respectively). Spindles can be obtained relatively free of cytoplasmic debris (Fig. 1), and a typical preparation from 4 × T-175 cm^2 flasks of cells usually yields approximately 1–2 × 10^7 spindles. The isolated spindles are only moderately immunogenic when injected into mice. Of 26 cell fusions carried out using spleenocytes of mice immunized with spindles, 41 hybridoma culture wells were initially found by immunofluorescence to contain antibodies that recognized microtubules and/or spindle components in fixed PtK$_2$ cells. The use of PtK$_2$ cells in immunofluorescence assays facilitated the observation of intracellular structures because they remain flat and well attached to the substrate during the cell cycle, including mitosis. Further analyses revealed that ~80% of the antibodies were antitubulins.

Among several interesting hybridoma clones obtained using these procedures, one (22MA2.1.1, hereafter designated 22MA2) was selected for use in the present study. The antibody was found to be of the IgM subclass by Ouchterlony double immunodiffusion of 22MA2 hybridoma supernatant against goat antibodies prepared against specific subclasses of mouse immunoglobulins. In this report we describe the use
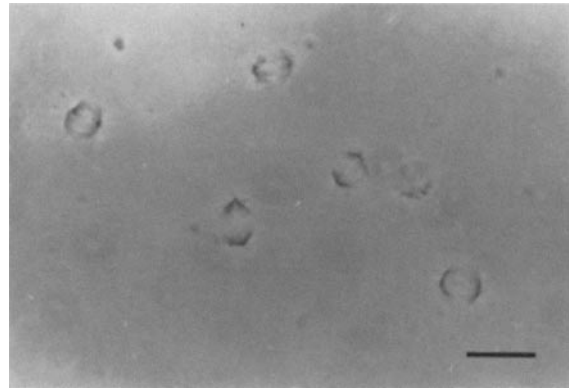

FIGURE 1 Phase-contrast micrograph of isolated HeLa spindles. Bar, 20 μm. × 450.

of the 22MA2 monoclonal antibody for the characterization of its antigen with respect to its intracellular distribution and some of its physical properties.

Intracellular Localization of the 22MA2 Antigen by Immunofluorescence

The immunofluorescence distribution of both tubulin and the 22MA2 antigen in the same interphase PtK$_2$ cell is shown in Fig. 2. The rhodamine-labeled rabbit antitubulin fluorescence pattern (Fig. 2a) shows a positively stained cytoplasmic microtubule complex, which is in agreement with previous reports (3, 34). The fluorescein-labeled 22MA2 antibody distribution (Fig. 2b) is co-localized with cytoplasmic microtubules, although with a lesser degree of antibody staining intensity. This relatively attenuated amount of 22MA2 staining of cytoplasmic microtubules (as compared with that of antitubulin) was consistently observed in this and all other immunofluorescence assays using the monoclonal antibody. Fig. 2 also shows that the 22MA2 antibody intensely stains the interphase nuclei of cells. The 22MA2 fluorescence is sharply defined by the nuclear envelope, and its intranuclear distribution appears homogeneous when examined by focusing through the nucleus. Fixed cells incubated with rabbit and mouse preimmune control sera (followed by incubation with rhodamine anti-rabbit IgG and fluorescein anti-mouse IgG + IgM, respectively) are shown in Fig. 2, c and d, and are negative.

The immunofluorescence staining pattern of the 22MA2 antibody during mitotic stages is shown in Fig. 3. At prophase (Fig. 3a) the 22MA2 labeling remains within the nucleus and is distributed throughout the nucleoplasm, in contrast to the condensing prophase chromatin. Some staining is seen associated with the separating centrosomes, and a relatively small amount is associated with the microtubules radiating from the centrosomes. Fig. 3b shows that at prometaphase, upon breakdown of the nuclear envelope, the newly forming spindle microtubules are intensely stained with the antibody. This abundance of spindle fiber staining with the 22MA2 antibody persists through metaphase (Fig. 3c) and anaphase when, in addition, staining of the continuous microtubules between the separating chromosomes can be seen (Fig. 3d). At telophase (Fig. 3e) the antibody stains the spindle remnants contained in the midbody, and the newly forming telophase nuclei show that the material recognized by the antibody is sequestered within the nucleus at this stage. Fig. 3f shows the
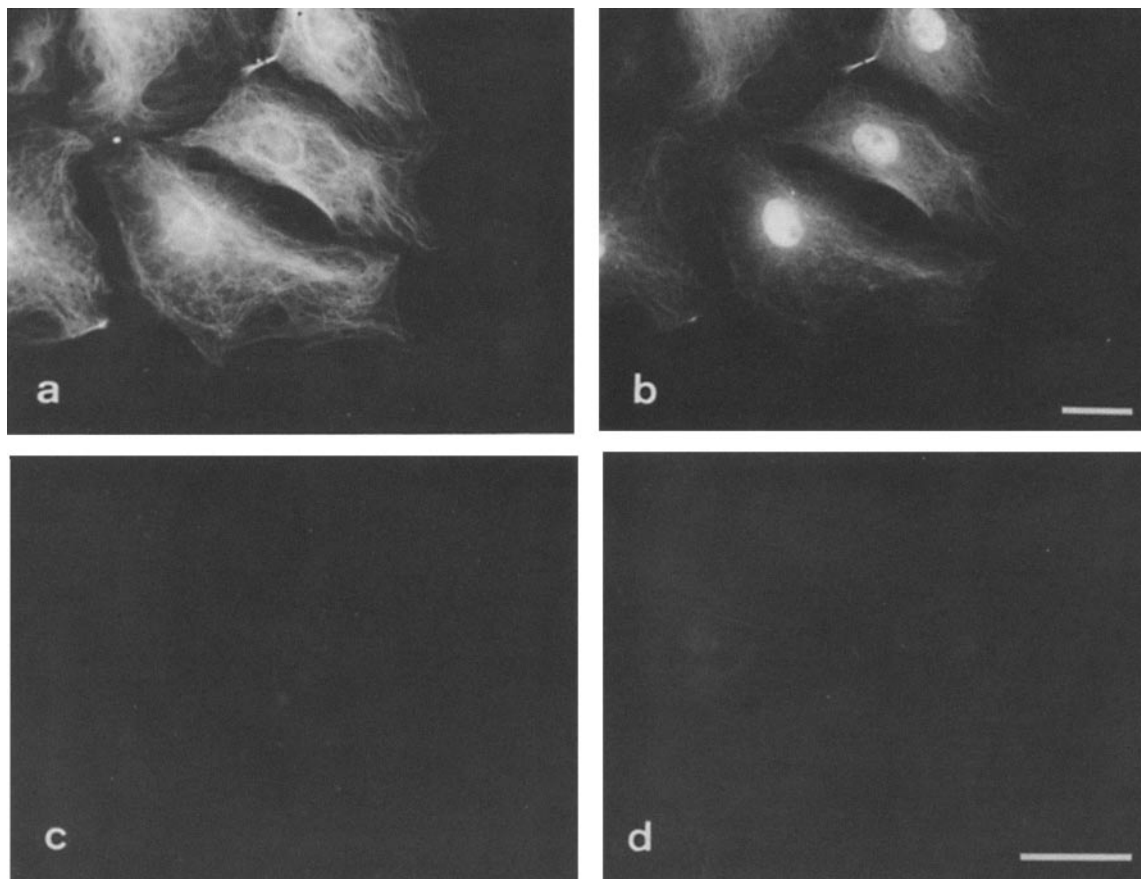
FIGURE 2 Double-label immunofluorescence pattern of antitubulin and 22MA2 monoclonal antibody staining in the same cell. PtK₂ cells were fixed and incubated with 22MA2 antibody followed by fluorescein goat anti-mouse IgG + IgM and then rabbit antitubulin followed by rhodamine goat anti-rabbit IgG. Photomicrographs were then taken at 1-min exposures using (a) rhodamine excitation for antitubulin and (b) fluorescein excitation for 22MA2 monoclonal antibody. (c) Preimmune rabbit serum control and (d) preimmune mouse serum control, both performed as described in the text. Bars, 20 μm. (a and b) × 460; (c and d) × 730.
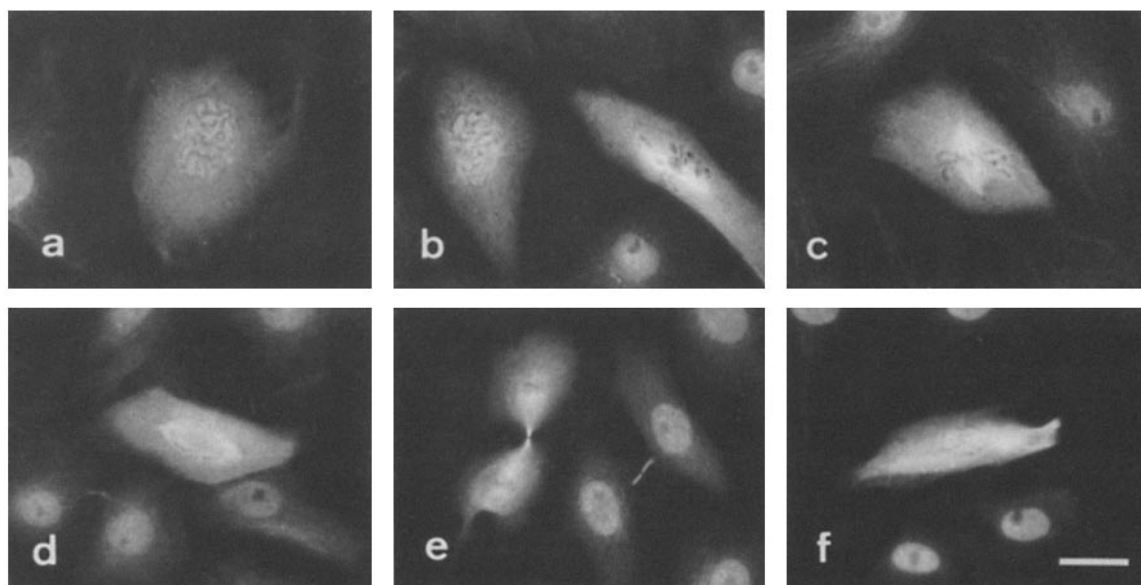

FIGURE 3 Immunofluorescence distribution of the 22MA2 antibody during mitosis. PtK₂ cells were processed for 22MA2 immunofluorescence as described in Materials and Methods. (a) Prophase cell; (b) prometaphase cell; (c) metaphase cell; (d) anaphase cell; (e) telophase cell. (f) PtK₂ cells treated with 1.3×10^{-7} M colcemid for 1 h at 37°C followed by fixation and processing for 22MA2 immunofluorescence. Bar, 20 μm. × 460.
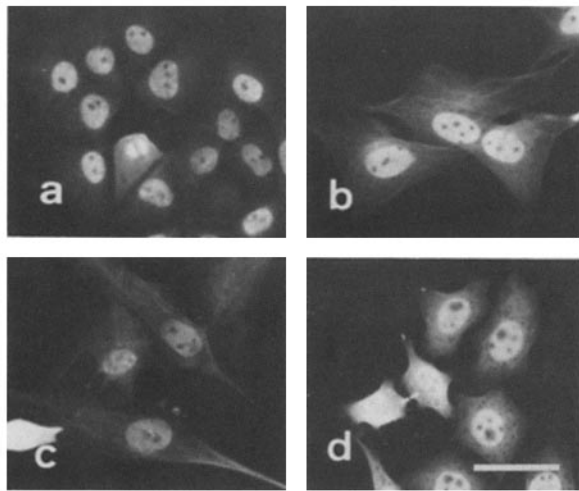
FIGURE 4 Immunofluorescence staining pattern of 22MA2 antibody in different cell types. Various cell types were fixed and processed for 22MA2 immunofluorescence as described. (a) BRL-3A (rat epithelium) cells; (b) BHK-21 (hamster fibroblast) cells; (c) WI-38 (human fibroblast) cells; (d) HeLa (human carcinoma) cells. Bar, 20 μm. × 550.

22MA2 fluorescence distribution in cells treated with colcemid. It can be seen that the spindle fiber staining is sensitive to colcemid in a manner similar to that of microtubules.

Fig. 4 shows the subcellular distribution of 22MA2 immunofluorescence in various cultured cell species. Interphase rat, hamster, and human cells all show the same general immunofluorescence pattern as that described above for PtK₂. Fig. 4a shows a metaphase BRL-3A cell whose mitotic spindle is positively stained with the 22MA2 antibody. The positive spindle staining was observed in the mitotic cells of all other species studied as well (micrographs not shown).

22MA2 Immunofluorescence Using Different Fixation Procedures

Because the 22MA2 antigen displays an unusual pattern of intracellular distribution, we decided to examine its immunofluorescence pattern in cells fixed using different methods. This was done in an attempt to determine whether the routine formaldehyde fixation used in the experiments shown in Figs. 2–4 could lead to an artifactual association of the 22MA2 antibody and/or antigen with nuclei and microtubules. PtK₂ cells were fixed by two additional methods; (a) cells were fixed with glutaraldehyde followed by borohydride treatment according to Weber et al. (35) or (b) cells were fixed using cold (−20°C) methanol (25). After fixation, cells were processed for immunofluorescence with the 22MA2 antibody in the usual manner.

Fig. 5 shows that both glutaraldehyde (Fig. 5a) and methanol (Fig. 5b) fixations result in positively stained spindles and nuclei using the 22MA2 antibody. However, methanol fixation gives poor preservation of cytoplasmic microtubule staining and results in the severe extraction of nuclei which show only a residual speckled appearance.

Biochemical Analyses of the 22MA2 Antigen

Electrophoretic analyses were undertaken in an attempt to identify the polypeptide molecular weight of the 22MA2 antigen and correlate its distribution in subcellular fractions

with the results of the immunofluorescence studies described above. Crude HeLa proteins from whole cells, cytoplasm, nuclei, and mitotic apparatuses were prepared and analyzed with SDS PAGE as described in Materials and Methods. Electrophoretic transfers (Western blots) were prepared from the gels and were immunochemically probed with the 22MA2 monoclonal antibody.

Fig. 6 shows that the antibody recognizes a single polypeptide band which exhibits a molecular weight of ~240,000. (In other experiments [not shown] 12.5% polyacrylamide gels
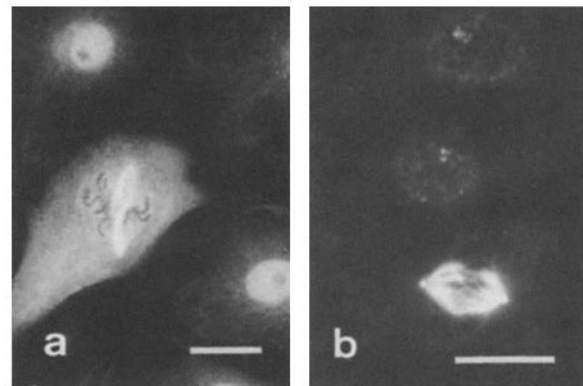

FIGURE 5 22MA2 immunofluorescence staining pattern in PtK₂ cells using different fixation procedures. Cells were fixed with either (a) glutaraldehyde or (b) cold methanol as described in Materials and Methods and processed for 22MA2 immunofluorescence. Bars, 20 μm. (a) × 460; (b) × 660.
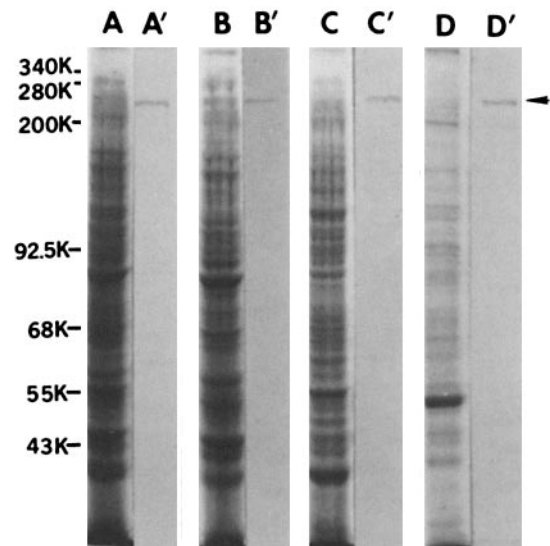

FIGURE 6 Immunochemical analysis of 22MA2 antigen distribution in HeLa subcellular extracts. HeLa whole cells or subcellular extracts were prepared and analyzed by SDS PAGE using an 8% polyacrylamide gel. Duplicate SDS PAGE protein patterns were then either fixed and stained with Coomassie Blue (lanes A–D) or electrophoretically transferred onto nitrocellulose and immunochemically analyzed with the 22MA2 antibody (lanes A'–D') as described in Materials and Methods. (A, A') Whole HeLa cells, 50 μg; (B, B') HeLa cytoplasmic fraction, 50 μg; (C, C') HeLa nuclear fraction, 50 μg; (D, D') HeLa mitotic spindles, 20 μg. Numbers on the left side refer to the electrophoretic migration of molelcular weight standards (K, 1,000): MAP-1 (340,000); MAP-2 (280,000); myosin heavy chain (200,000); phosphorylase b (92,500); BSA (68,000); tubulin (55,000); ovalbumin (43,000). The arrowhead denotes the position of the 22MA2 antigen.

were used in similar analyses to determine whether the 22MA2 antibody recognizes any lower molecular weight components [≥10,000 mol wt], and none were detected.) The protein loads of each of the crude extracts in lanes A', B', and C' were roughly equivalent at 50 µg each, and two sets of the samples were run on the same gel. One set was cut from the gel and stained with Coomassie Blue (lanes A, B, and C), while the other set of samples was electrophoretically transferred onto the same sheet of nitrocellulose and simultaneously probed with the antibody (lanes A', B', and C', respectively). Therefore the relative antibody staining intensity of the bands should correspond to the quantity of antigen present as a relative percentage of the total protein in the samples. Using this as a frame of reference, we found that the relative amounts of antigen present in the cytoplasmic and nuclear fractions (lanes B' and C', respectively) correspond well with the results of the immunofluorescence studies described above. It should be noted that the bands recognized by the 22MA2 antibody in the nuclear (lane C') and mitotic apparatus (lane D') fractions co-migrate at 240,000 mol wt.

Similar analyses were performed using whole-cell extracts from several species of cultured cells, and the results are shown in Fig. 7. In addition, rabbit brain microtubule protein (isolated by two cycles of assembly/disassembly according to Borisy et al. [1] and kindly provided by Dr. Lou Morejohn, University of California, Irvine) was analyzed to determine any cross-reactivity of the 22MA2 antibody with the high molecular weight neural MAPs. The polypeptide recognized by the 22MA2 antibody exhibits a molecular weight of 240,000 in various species ranging from marsupial to human. Fig. 7 (lanes E and E') also shows that the 22MA2 antibody does not cross-react with brain tubulin or the high molecular weight MAPs using the Western blot procedure.

In several initial experiments of the type described immediately above, the antibody detected a single polypeptide with a molecular weight of 115,000 (26). These electrophoretic analyses were performed using nuclei or mitotic spindles that were isolated in the presence of the protease inhibitor apro-
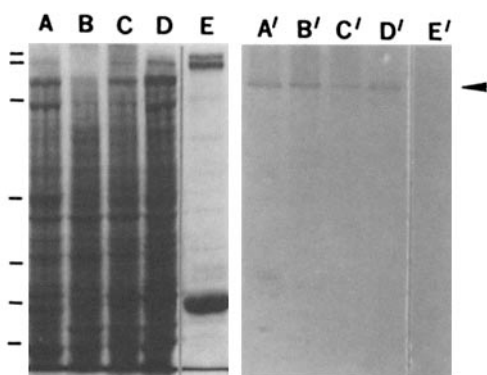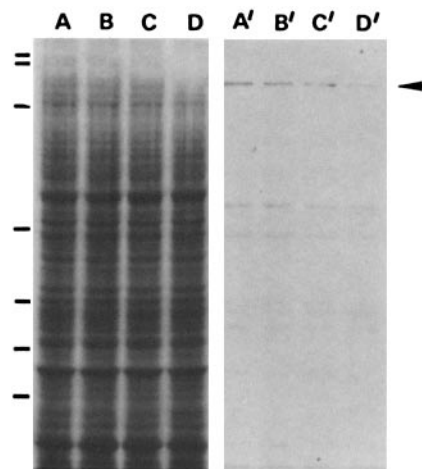

FIGURE 8 Effects of protease inhibitors on the preservation of the 22MA2 antigen in cell extracts. HeLa cells were disrupted and the nuclei were isolated in the presence of (A, A') aprotinin; (B, B') PMSF; (C, C') pepstatin A + leupeptin; or (D, D') no inhibitor, as described in the text. Duplicate samples (all 50 µg) were analyzed by SDS PAGE (7.5% gel) and either Coomassie Blue-stained (lanes A–D) or electrophoretically transferred onto nitrocellulose and probed with the 22MA2 antibody (lanes A'–D'). Markers on the left refer to molecular weight standards as in Fig. 6. The arrowhead denotes the position of the 22MA2 antigen.

tinin (see Materials and Methods) and had been stored at either 4° or −20°C in their respective isolation buffers for 1–5 d prior to electrophoretic analysis. Later experiments using samples that were freshly isolated and immediately electrophoresed showed the appearance of the 240,000-mol-wt polypeptide. Therefore we attempted to examine the effectiveness of other protease inhibitors in preventing the degradation of the 240,000-mol-wt antigen during the isolations. The results of such an experiment are shown in Fig. 8. This figure shows the SDS PAGE and Western blot antibody analysis of extracts prepared from HeLa cells in the presence (and absence) of several protease inhibitors. We accelerated any proteolysis that occurred during extractions by allowing the extracts to stand for 2 h at 23°C before boiling them in electrophoresis sample buffer. Subsequent analysis showed that considerable proteolysis of the 240,000-mol-wt antigen occurs even in the presence of aprotinin, PMSF, and pepstatin A + leupeptin (Fig. 8, lanes A', B', and C', respectively). Several discrete proteolytic fragments of the 240,000-mol-wt antigen are recognized by the 22MA2 antibody, and occur at molecular weights of ~115,000 and 95,000, and a cluster at ~68,000. Also, lane D' of this figure shows that almost no antigen is preserved when extracts are prepared in the absence of any protease inhibitors. Comparisons of Fig. 8 lanes A'–D' show that protease inhibitors do contribute to the preservation of the 240,000-mol-wt antigen, and, in decreasing order of relative effectiveness, are aprotinin > PMSF > pepstatin A + leupeptin > none. We must therefore conclude that the 115,000-mol-wt peptide detected in previous experiments was a discrete proteolytic fragment of the 240,000-mol-wt antigen, and occurred upon cold storage of extracts even in the presence of aprotinin. MAPs have been observed to be susceptible to proteolysis (29, 33), and the 240,000-mol-wt antigen, as a putative MAP, appears to be no exception. Therefore in all subsequent experiments (such as those previously shown in Figs. 6 and 7), extracts were prepared using a combination of

FIGURE 7 Immunochemical analysis of 22MA2 antigen in various cell species. Whole cell extracts were prepared from different cell types and analyzed by SDS PAGE using a 6.5% polyacrylamide gel. Duplicate electrophoresed samples were then either stained with Coomassie Blue (lanes A–D) or electrophoretically transferred onto nitrocellulose and analyzed with the 22MA2 antibody (lanes A'–D'). (A, A') PtK$_2$ extract, 40 µg; (B, B') 3T3 extract, 40 µg; (C, C') rabbit fibroblast extract, 40 µg; (D, D') WI-38 extract, 40 µg; (E, E') rabbit brain microtubule protein, 10 µg. Markers on the left refer to molecular weight standards as in Fig. 6. The arrowhead denotes the position of the 22MA2 antigen.

aprotinin and pepstatin A and, more importantly, were immediately boiled in electrophoresis sample buffer before storage at −20°C.

Immunofluorescence Distribution of the 22MA2 Antigen in Confluent Cells

Our early immunofluorescence studies using the 22MA2 antibody occasionally showed stained cells that were negative with respect to the nuclear fluorescence described in Figs. 2–4 above. Upon reexamination of these specimens, we observed that the coverslip cultures used in those studies had been allowed to overgrow before fixation. This suggested that the 22MA2 antigen might no longer be present in nuclei of cells that either had reached plateau phase in response to confluence (were no longer dividing) or had depleted the culture medium. Immunofluorescence experiments were then carried out to determine which of these two conditions might result in the absence of nuclear fluorescence with the 22MA2 antibody.

PtK$_2$ and BHK-21 cells were plated on coverslips either (a) at high initial density (100% confluence) in fresh MEM containing 10% FCS and were incubated for 24–36 h, or (b) at low density (≤25% confluence) in MEM containing 0.1% FCS and were.incubated for 48–72 h. Coverslips were then fixed and processed for 22MA2 immunofluorescence in the usual manner. The results of these experiments are shown in Fig. 9. Fig. 9, a and b clearly indicates that in 100% confluent cells, although the slight staining of the cytoplasmic microtubules remains, the nuclei are devoid of any staining with the antibody (compare with Figs. 2–5). Immediately prior to fixation, parallel coverslip cultures examined by phase-contrast microscopy and trypan dye exclusion appeared perfectly
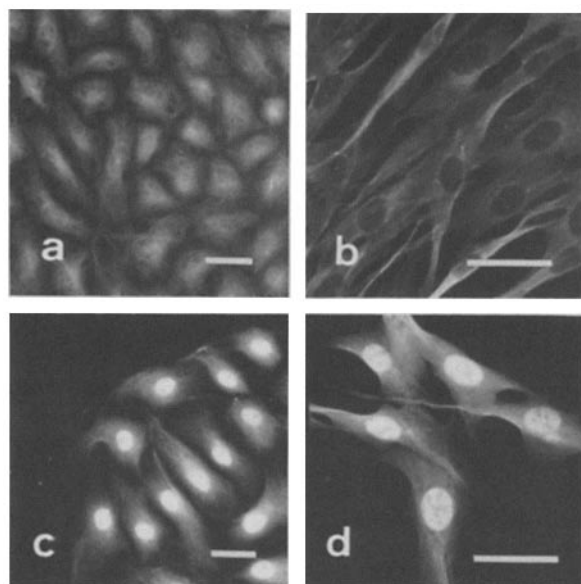
FIGURE 9   Immunofluorescence distribution of the 22MA2 antigen in confluent cells. PtK$_2$ (a and c) and BHK-21 (b and d) cells were cultured at 100% confluence in MEM + 10% FCS (a and b) or ≤25% confluence in MEM + 0.1% FCS (c and d) and were fixed and processed for 22MA2 immunofluorescence as described in the text. Both cell types display negative immunofluorescent staining of nuclei at 100% confluence (a and b), but they show positively stained nuclei at subconfluence even in the presence of low serum (c and d). Bars, 20 μm. (a and c) × 300; (b and d) × 550.
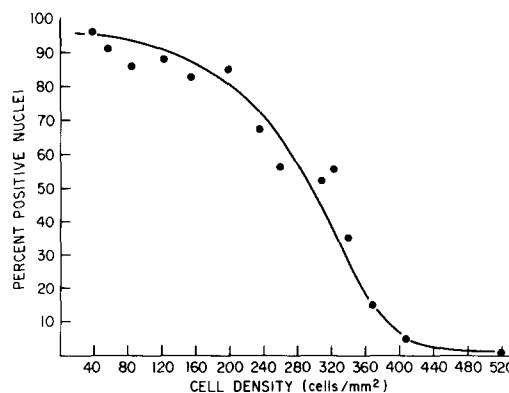
FIGURE 10   Quantitative correlation of cell density with 22MA2 nuclear staining. BHK-21 coverslip cultures were grown at various densities for 24–48 h and then fixed and processed for 22MA2 immunofluorescence. Each specimen was then tallied for total cells in each area examined by phase-contrast microscopy (usually 5 mm^2 per area counted) and also for the number of cells displaying positively stained nuclei in that same area as seen with fluorescence optics. Results are expressed as total cells counted per square millimeter (abscissa) vs. percentage of cells displaying positive fluorescent nuclei (ordinate).

viable and healthy (results are not shown here). This experiment also was carried out using 3T3, BRL-3A, WI-38, and HeLa cells and the results were the same as those shown in Fig. 9 for PtK$_2$ and BHK-21 cells. Fig. 9, c and d shows that cells plated sparsely in medium containing low serum for up to 72 h still display positive nuclear fluorescence with the 22MA2 antibody. Taken together these results suggest that the absence of nuclear fluorescence is correlated with high cell density rather than depletion of the growth medium.

Growth curve experiments were then carried out to determine whether the 22MA2 nuclear fluorescence was correlated with cell density at various stages of culture confluence. BHK-21 cells were plated at various densities and allowed to grow for 24–48 h. Coverslips were then fixed and processed for 22MA2 fluoresence, and quantitative counts were taken of the percentages of cells displaying positively fluorescent nuclei as a function of cell density. Fig. 10 shows that almost 100% of BHK-21 cells display positive nuclear fluorescence at low densities, but that the percentage of positive nuclei gradually declines as the cells grow to confluence. At confluence, the nuclei are no longer stained by the antibody. This same general phenomenon was observed in all the other cell lines used in this type of study, and the results indicate that the percentage of cells showing positive nuclear fluorescence is inversely proportional to the density of the cell culture.

DISCUSSION

Hybridoma antibodies have proved to be invaluable as a means of "fishing" for previously unidentified molecules that are associated with specific cellular functions, and especially for molecules for which no purification method exists (for review see reference 11). The present report, using a combination of existing methodologies, outlines a procedure whereby monoclonal antibodies can be produced against specific molecules associated with the mitotic apparatus of cultured mammalian cells. In our hands this experimental approach has produced several monoclonal antibodies of particular interest.

Because the immunizations in this study consisted of HeLa (human) spindles and the hybridomas were screened using fixed PtK$_2$ (marsupial) cells, this procedure selects for those antibodies that are likely to cross-react with their specific antigens in a wide variety of mammalian species. This is in contrast to the results of other investigators who have prepared antibodies against MAPs or spindle components (also using extracts from human cells) that recognize only human and/ or primate forms of the antigens (5, 12, 13, 18). However, while our procedure selects for antibodies that might be more utilitarian for a variety of purposes, it could also have the drawback that in the screening procedure some desirable hybridomas might go undetected because they produce antibody that reacts only with the human form of the antigen.

In this report we have described the production of a particular monoclonal antibody and its use in the characterization of a somewhat unusual putative microtubule-associated protein. The possibility that this antigen is an MAP is supported by results that show that the 22MA2 monoclonal antibody intracellularly co-localizes with both mitotic spindles and (to a lesser extent) cytoplasmic microtubules, and that this distribution is colcemid sensitive. It is also shown that the antibody immunofluorescently stains the nuclei of interphase cells in the exponential growth phase. The results of the fixation tests suggest that methanol is an inferior fixative with regard to the 22MA2 antigen, and could lead to the possible misinterpretation that the 22MA2 antigen is a strictly spindle-specific MAP. We cannot make this conclusion because of the slight but distinct staining of cytoplasmic microtubules with the aldehyde fixation methods. The immunofluorescent images seen using the 22MA2 antibody with methanol-fixed cells are somewhat similar to those reported by other investigators (12) using an antibody against a 200,000-mol-wt HeLa MAP in immunofluorescence procedures also using methanol-fixed cells. In that study the authors suggested that the 200,000-mol-wt MAP is a spindle-specific component, but did not indicate whether the same staining pattern was observed using different fixation methods.

The present report also shows that the 22MA2 antibody recognizes a single 240,000-mol-wt polypeptide whose relative distribution in subcellular extracts agrees with the results of the immunofluorescence studies. In this regard, the antibody could be recognizing a similar determinant on two functionally unrelated molecules which happen to have the same molecular weight. While such a series of coincidences is not inconceivable, it seems highly improbable. In this study, we have also shown that the antibody recognizes the 240,000-mol-wt polypeptide in extracts from various cells ranging from marsupial to human. Here the results again agree with the immunofluorescence studies, in that the antibody recognizes an antigenic determinant that appears to have been conserved through the course of mammalian evolution.

Investigators using various methods have reported the identification of a number of mammalian non-neuronal MAPs in the molecular weight range of 200,000–300,000 (4, 12, 13, 24, 33). The absence of the 240,000-mol-wt MAP in microtubule proteins prepared by assembly/disassembly methods (4, 24, 33) could be because it does not co-assemble with microtubules in vitro, or becasuse the elevated temperatures required for microtubule assembly in vitro may result in the proteolytic degradation of the 240,000-mol-wt MAP. The latter possibility is very likely in view of the proteolysis studies presented here. Whether any of the previously reported MAPs

might be proteolytic fragments of the 240,000-mol-wt protein described in the present study (or vice versa) cannot be ascertained from the present data. Vallee and Bloom (32) have recently identified a 235,000-mol-wt spindle-associated MAP in extracts from sea urchin eggs, but their data do not indicate the nature or distribution of this antigen in other cell types. Further studies in this area might reveal some interesting relationships between the various high molecular weight MAPs which have been reported.

Investigators have previously speculated that certain factors involved in mitotic spindle functions might be sequestered within the nucleus of cells during interphase (27). More recent studies have identified specific nonchromosomal proteins which appear to be associated with the mitotic apparatus and the interphase nucleus (12, 18). Leyderson and Pettijohn (18) identified a 300,000-mol-wt protein which is associated with the nuclear matrix during interphase and the poles of the mitotic apparatus during mitosis. Izant et al. (12) provided evidence for a 200,000-mol-wt MAP which is associated with interphase nuclei and mitotic spindle fibers. Thus evidence is accumulating that suggests that a general class of spindle-associated proteins/regulatory factors exists which are sequestered (at least to some extent) within the nucleus until needed during cell division. The results of our biochemical and immunocytochemical analyses suggest that the 240,000-mol-wt 22MA2 antigen may be included in such a class of proteins.

An intriguing characteristic of the protein seen in the present report is that it is no longer detectable in the nuclei of confluent cells by immunofluorescence using the 22MA2 antibody. Several possible reasons for this phenomenon can be envisioned, among which are: (*a*) the protein may no longer be sequestered in nuclei; (*b*) the protein may undergo modification at the 22MA2 antibody binding site; or (*c*) the protein may become complexed with another macromolecule at the antibody binding site. Any of these phenomena could be occurring in response to cellular processes/signals involved in the density-dependent "turning off" of a cell with respect to its immediate potential for replication. This is, however, no more than speculation derived from general observations. The possible function of the 240,000-mol-wt protein can only be elucidated through further study. Nevertheless, the possibility that this protein changes (with regard to either distribution, structure, or association with other molecules) in response to the cell's potential for growth/division is potentially very interesting. Whatever the reason for the phenomenon, the search for its cause ideally might lead to new data regarding the mechanisms that determine the division potential of the cell. For this reason, we are currently engaged in a variety of studies toward this end.

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