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Identification of the 190 KD Microtubule-Associated Protein in Cultured Fibroblasts and Its Association with Interphase and Mitotic Microtubules.

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ABSTRACT. We previously investigated the biochemical characteristics of microtubule-associated proteins (MAPs) of the adrenal medulla and adrenal cortex and found that they contain a new kind of MAP with a molecular weight of 190,000 (190 kD MAP) as a major species (Kotani, S., H. Murofushi, S. Maekawa, C. Sato, and H. Sakai. *Eur. J. Biochem.* 156, 23–29, 1986). We now have used an affinity purified anti-(190 kD MAP) antibody and show by indirect immunofluorescent microscopy the association of this MAP with microtubules *in situ* in TIG-3 cells (human embryonic lung fibroblasts). The 190 kD MAP was present along the interphase and mitotic microtubules, and there was no marked difference between the staining pattern with anti-tubulin and that with anti-(190 kD MAP) antibodies, evidence that the localization of 190 kD MAP is not restricted to the subset of microtubules. We also isolated MAPs from TIG-3 cells and identified their 190 kD MAP as a major heat-stable component. Several other unidentified polypeptides were recovered in the MAP fraction specifically.

Microtubules are believed to have diverse roles in such processes in eukaryotic cells as mitosis, secretion and intracellular transport (15). Tubulin, the major component of microtubule is a conserved protein in species and in tissues, therefore, the diversity of microtubule functions is believed to be due to the diversity of accessory proteins (microtubule-associated proteins; MAPs) present (39).

In spite of the "one MAP-one function" hypothesis presented by Vallee *et al.* (39), so far relatively few MAPs have been characterized. Moreover, the source of mammalian MAPs was restricted to neuronal cells (brains) (11, 17, 19, 23, 31) or non-neural cultured cell lines (8, 12, 25, 28, 40). Untill recently, attempts to identify MAPs in non-neural organs ended in failure (2, 10, 32, 37).

We reported elsewhere the isolation of MAPs from non-neural adrenal gland cells (22) and showed that the major MAPs of the adrenal medulla are high molecular weight proteins that are almost identical to MAP1 and MAP2, and that the adrenal

Abbreviations used: MAPs, microtubule-associated proteins; PBS, phosphate-buffered saline (20 mM sodium phosphate pH 7.2, 150 mM NaCl); FCS, fetal calf serum; FCS-PBS, PBS containing 5% FCS; SDS, sodium dodecyl sulfate, SDS-PAGE, polyacrylamide gel electrophoresis in the presence of SDS.

gland contains another MAP of 190,000 mol wt (the 190 kD MAP) as a major species. Subsequently, we purified this 190 kD MAP from bovine adrenal cortex to homogeneity (27). The purified 190-kDa MAP clearly differed in several aspects from MAP1, MAP2 and tau of mammalan brain, but it met the criteria for a MAP in its promotion of the assembly of tubulin and its binding to microtubules *in vitro*. Another important characteristic of MAPs, the association with microtubules *in situ*, could not be proved.

We used indirect immunofluorescent microscopy to investigate this association. First, we isolated the MAP fraction from cultured human embryonic lung fibroblasts (TIG-3 cells) and confirmed that a protein similar to 190 kD MAP is present in those cells. Cell specimens then were fixed and stained with affinity purified anti-(190 kD MAP) antibody and with anti-tubulin monoclonal antibody. The association of 190 kD MAP with interphase and mitotic microtubules is shown, and conclusive evidence is given that the 190 kD polypeptide is a microtubule-associated protein.

MATERIALS AND METHODS

Chemicals. Taxol was the gift of the Natural Product Branch, Division of Cancer Treatment, National Cancer Institute (USA). It was dissolved in dimethyl sulfoxide to a stock concentration of 1 mM then stored at −80 °C. Rhodamineconjugated phaloidine was purchased from Molecular Probes Inc. (USA), goat IgG from Sigma Chemical Co. (USA) and Triton X-100 from Nakarai Chemicals Ltd. (Kyoto). All other materials used were of reagent grade.

Cells and antibodies. TIG-3 cells (human embryonic lung fibroblasts) were separated from the lungs of a female embryo and maintained as described elsewhere (26). HeLa cell strain S3 was the gift of Dr. T. Shenshu (Tokyo Metopolitan Institute of Gerontology). A431 cells were given by Dr. I. Yahara (Tokyo Metropolitan Institute of Medical Science) and PC12 cells by Dr. T. Amano (Mitubishi-Kasei Institute of Life Science).

Anti-MAP1, anti-MAP2, anti-tau and anti-(190 kD MAP) antisera were obtained as described elsewhere (22). Anti-(190 kD MAP) antibody was affinity purified from rabbit anti-(190 kD MAP) antiserum by the method of Olmsted (30). Anti-tubulin monoclonal antibody (YOL1/32) was purchased from Sera-Lab (UK) and the second antibodies (fluorescein isothiocyanate-conjugated goat anti-rabbit IgG antiserum, rhodamin isothiocyanate-conjugated goat anti-rat IgG antiserum and horseradish peroxidase-conjugated goat anti-rabbit IgG antiserum) from Cappel laboratories (USA).

Protein preparations. Taxol-dependent isolation of MAPs from TIG-3 cells was done by the method of Vallee (38) with the modifications described (22). Proteins were heat treated by the method of Fellous *et al.* (16). The purification procedure used for the 190 kD MAP is given (27). The extracts from cultured cells used for immunoblotting were obtained by dissolving the cells directly in the electrophoresis sample buffer that contained sodium dodecyl sulfate (SDS) then boiling them for 5 min.

Indirect immunofluorescent microscopy. TIG-3 cells were grown on glass coverslips in 30-mm petri dishes. Before fixation, the cells were washed for 10 min in phosphate-buffered saline (PBS) containing 5% fetal calf serum (FCS-PBS) to reduce non specific binding, after which they were fixed with 3.7% formaldehyde for 10 min at 37 °C. After extensive washing in PBS, the cells were rendered permeable by 0.5% Triton X-100 for 10 min then washed twice in PBS at room temperature. The now permeable cells were blocked for 30 min at 37 °C with FCS-PBS containing 0.1% goat IgG, then washed briefly in PBS and incubated

with the first antibodies for 30 min at 37°C. These cells were then washed three times in PBS and incubated for 30 min at 37°C with the second antibodies diluted in FCS-PBS that contained 0.1% goat IgG. Observations were made with a Nikon Fluophoto fluorescence microscope. The microscopic images were recorded with a photomicrographic attachment (Nikon M-35FA) on Kodak Tri-X film (400 ASA).

Other methods. Polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE) followed the method of Laemmli (24). The gels obtained were stained with Coomassie Brillant Blue R-250. Immunoblotting was done by the method of Towbin *et al.* (36).

RESULTS

Isolation of TIG-3 MAPs. Before doing the immunofluorescent microscopy, we immunoblotted the extracts of various cells to see whether those cells contain protein species immunoreactive with the anti-(190 kD MAP) antiserum. We found that several; TIG-3, HeLa, PC 12 and A431 cells, contain immunoreactive species whose mobilities in SDS-PAGE are almost identical to that of the 190 kD MAP.

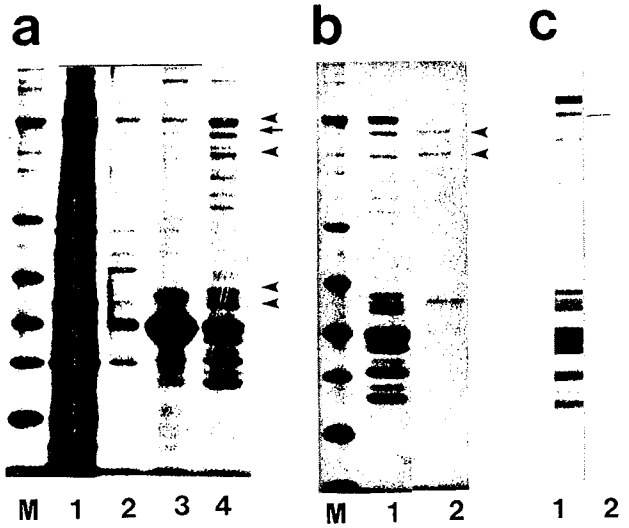

Fig. 1. a, Polyacrylamide gel electrophoresis of protein fractions made at each step in the preparation of the MAPs from TIG-3 cells. Microtubules were formed from cytosolic extracts of TIG-3 cells and were collected by centrifugation. 1, microtubule-depleted supernatant; 2, supernatant after the microtubules were washed in taxol-containing buffer solution; 3, tubulin-containing pellets and 4, MAP-containing supernatant after a wash in buffer solution containing taxol and 0.35 M NaCl. 5 μl of 1 and 50 μl each of 2, 3 and 4 were electrophoresed. The arrow indicates the protein with a mobility similar to that of bovine adrenal 190 kD MAP. The arrowheads indicate the major proteins in the MAP fraction. Several species, substantial amounts of which were recovered in the tubulin fraction (3), are not marked. b, Heat treatment of the MAP fraction. The MAP fraction identical to a-4 was electrophoresed before (1) and after (2) heat-treatment. Heat-stable components are indicated by the arrowheads. c, Immunoblotting of the TIG-3 MAP fraction with anti-(190 kD MAP) antibody. Protein staining (1) and immunostaining (2) of the nitrocellulose sheet. Lanes marked "M" represent marker proteins: myosin heavy chain (200,000), phosphorylase *b* (94,000), bovine serum albumin (68,000), tubulin (55,000), actin (42,000), glyceraldehyde-3-phosphate dehydrogenase (35,000) and carbonic anhydrase (29,000).

We chose the TIG-3 cells for our microscopy study because their immunoreactive species appeared to be the most abundant. Also, we further characterized the TIG-MAPs to make sure that the immunoreactive protein had the characteristics of a MAP. The MAP fraction isolated by the taxol method contained a protein band whose mobility was similar to that of the 190 kD MAP (Fig. 1). This protein is heat-stable and immunoreactive with anti-(190 kD MAP) antiserum (Fig. 1b and c). Judging from its mobility in SDS-PAGE, its heat-stability, its immunoreactivity and its association with microtubules, we conclude that the protein contained in TIG-3 cells is a protein similar to 190 kD MAP; therefore, TIG3 cells are useful for the study of the localization of 190 kD MAP in the cell. Immunoblotting studies done with anti-MAP1, anti-MAP2 and anti-tau antisera revealed that the TIG-MAP fraction contained no appreciable amounts of MAP1, MAP2 and tau (data not shown).

Indirect immunofluorescent microscopy. Results of double staining with the anti-(190 kD MAP) and anti-tubulin antibodies are shown in Fig. 2. Both the low and high magnification images show essentially complete correlations between the localization of tubulin and that of 190-kDa MAP. No correlation between the localizations of 190-kDa MAP and actin was found when TIG3 cells were stained with anti-(190 kD MAP) antiserum and rhodamine-conjugated phalloidin, a reagent that stains actin-containing stress fibres (data not shown).

The control experiments are shown in Fig. 3. When the anti-(190 kD MAP) anti-

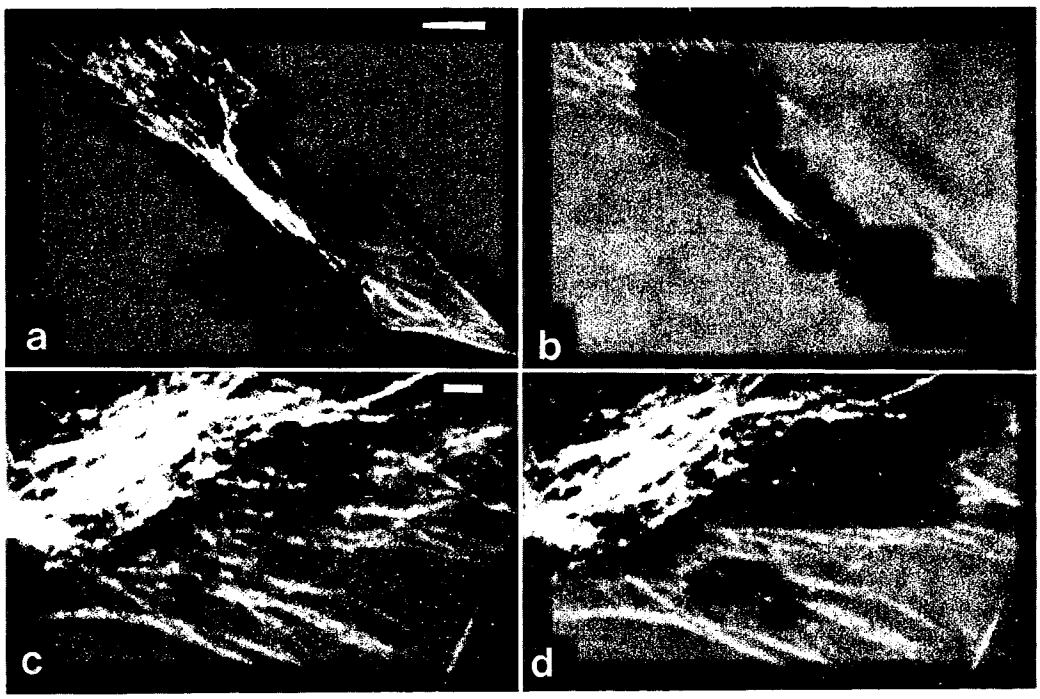

Fig. 2. Co-localization of 190 kD MAP and tubulin in interphase cells. Double-labeling was done with anti-tubulin monoclonal antibody (a, c) followed by rhodamin anti-rat IgG, and with rabbit anti-(190 kD MAP) antibody (b, d) followed by fluorescein anti-rabbit IgG. Bars: 10 μm in a, for a and b; 2 μm in c, for c and d.

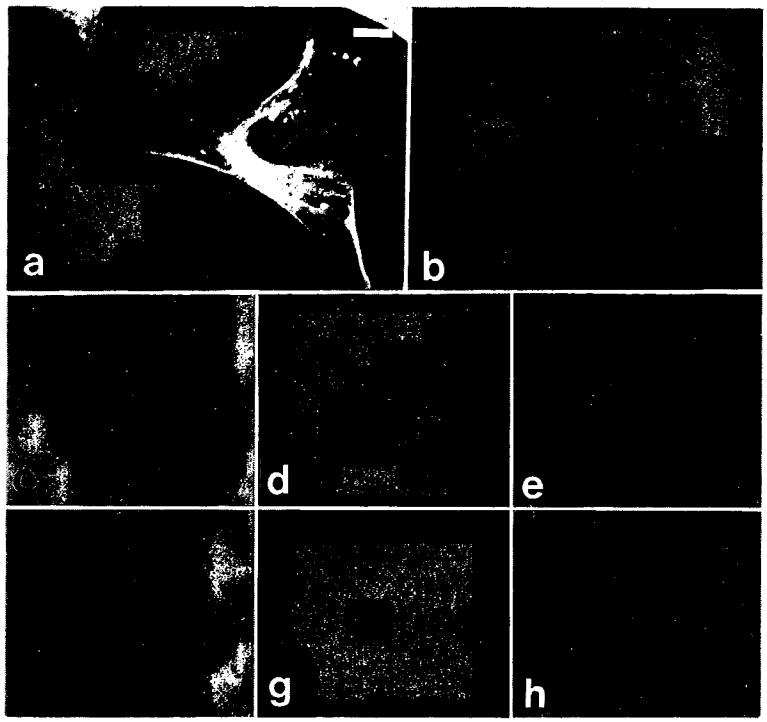

Fig. 3. Control experiments for the localization of 190 kD MAP. a and b, double labeling with anti-tubulin (a) and anti-(190 kD MAP) (b) antibodies as in Fig. 2 except that the anti-(190 kD MAP) antibody was blocked by incubating with purified 190-kDa MAP for 1 h at 37°C. c, d and e, microtubules were disrupted by colchicine treatment then stained with anti-tubulin (d) and anti-(190 kD MAP) (e) antibodies. c is a phase contrast micrograph. f, g and h, microtubules disrupted by cold treatment then stained with anti-tubulin (g) and anti-(190 kD MAP) (h) antibodies. f is a phase contrast micrograph. Bar: 10 μm for all the micrographs in this figure.

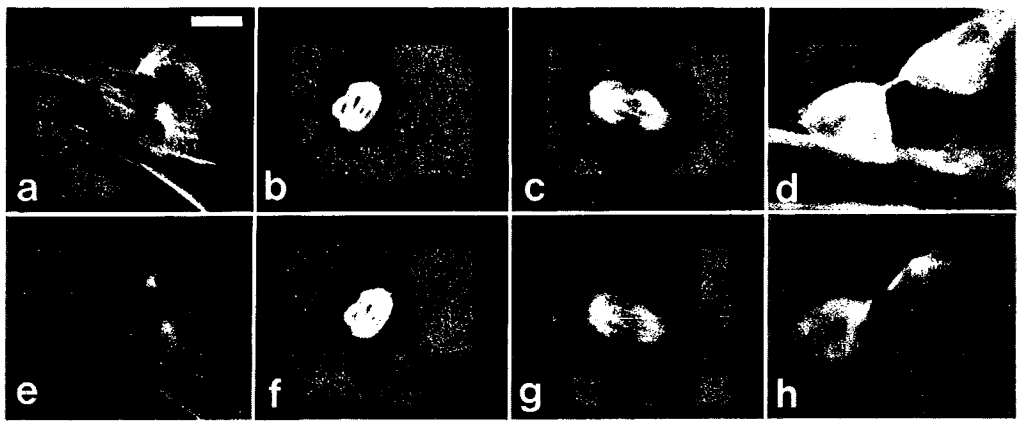

Fig. 4. Co-localization of 190 kD MAP and tubulin in mitotic cells. Double labeling was done with anti-tubulin (a, b, c, d) and anti-(190-kDa MAP) (e, f, g, h) antibodies as in Fig. 2. Cells that correspond approximately to prophase (a and e) metaphase (b and f) anaphase (c and g) telophase (d and h) are shown. Bar: 10 μm for all the micrographs in this figure.

body was blocked with purified 190 kD MAP for 1 h at 37°C, staining of 190 kD MAP was greatly reduced despite the clear staining of microtubules by the anti-tubulin antibody in the same cell (Fig. 3 a and b). Alternatively, when TIG3 cells were treated with colchicine for 2 h (Fig. 3 c, d and e), or were kept at 5°C during the serum-wash and fixation steps (Fig. 3 f, g and h), very faint, diffuse stainings were observed in both the tubulin and 190 kD MAP stainings. In the cold treatment, in particular, virtually no stainings were visible. These results are evidence that the depolymerized microtubule proteins are easily washed out under our fixation conditions.

The association of 190 kD MAP with mitotic microtubules is shown in Fig. 4. As with the interphase microtubules, 190 kD MAP was localized along the microtubules in all the mitotic phases viewed.

DISCUSSION

In the first half of this report, we characterized the MAPs of culture cells. The presence of 190 kD MAP-related species in several cell lines, especially in HeLa cells, strongly suggests a close relation between 190 kD MAP and HeLa cell MAP (8, 40). These cultured cell MAPs are now being isolated and characterized.

We successfully isolated TIG-3 MAPs (Fig. 1). The striking features of the MAP fraction are that at least five polypeptides with molecular weights of 200, 190, 150, 65 and 60 kD, are specifically recovered in this fraction; that two of them (190 and 150 kD) are heat-stable; that a heat-labile 200 kD polypeptide is the most prominent species; and that a protein related to 190 kD MAP is present, but that MAP1, MAP2 and tau are absent as judges from their mobilities in SDS-PAGE, their heat-stabilities and immunoreactivities. The 200 kD species may be related to the fibroblast MAP reported by Duerr *et al.* (14), but it is clearly distinct from HeLa cell MAP in its heat stability (41). The absence of MAP1 is in contrast to earlier reports (5, 21, 29, 33). To date, many published studies have dealt with fibroblast MAPs (12, 14, 21, 33), but the constituents of the MAPs differ from report to report partly because of the differences in the methods used and in the cell lines examined. Further characterization of each component at biochemical level is neccessary before a definitive characterization of fibroblast MAPs can be made.

Since the first report by Sherline and Shavonne (35), indirect immunofluorescent light microscopy has become an important technique with which to show the *in situ* association of a particular MAP with microtubules. By this technique, various MAPs were shown to associate with the microtubule (7, 13, 19, 31, 35) but some reseachers have argued against this association (1, 18, 34). Our present results show that the localization of 190 kD MAP is analogous to the localization of the previously reported MAPs. 190 kD MAP was shown to localize along interphase and mitotic microtubules in all the stages viewed. This localization did not appear to be restricted to the subset of microtubules, but the possibility cannot be ruled out that 190 kD MAP does associate only with the subset of microtubules in morphologically and functionally differentiated cells as reported for other MAPs (3, 4, 9, 20). Some MAPs are reported to associate with structures other than microtubules (1, 6, 34), but the only ordered structure that 190 kD MAP associates with appeares to be microtubules because double staining with phalloidin gave negative results and the ordered staining pattern was completely lost when microtubules were disrupted.

As reported elsewhere, 190-kDa MAP stoichiometrically stimulates the assembly of purified tubulin and binds to reconstituted microtubules *in vitro* (27), thereby meeting the criteria for a MAP. Now that its association with the cytoplasmic microtubules is definite, 190 kD MAP takes its place as a true member of the mammalian MAPs along with MAP1, MAP2 and tau.

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