Differential distribution of microtubule-associated proteins MAP-1 and MAP-2 in neurons of rat brain and association of MAP-1 with microtubules of neuroblastoma cells (clone N$_2$A)

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To study the individual location of the microtubule proteins MAP-1 and MAP-2 in neuronal tissues and cells, antisera to electrophoretically purified MAP-1 and MAP-2 components were raised in rabbits. When frozen sections through rat brain were examined by immunofluorescence microscopy the ratios through repeated cycles of in vitro polymerization/depolymerization, have attracted considerable interest. Important features of these proteins are their ability to stimulate microtubule polymerization in vitro (Murphy and Borisy, 1975; Weingarten et al., 1975; Sloboda et al., 1976; Kuznetsov et al., 1981) and the potential of high mol. wt. MAPs to form sidearms on microtubules (Murphy and Borisy, 1975; Dentler et al., 1975; Amos, 1977). It is an intriguing possibility, therefore, that microtubules with potentially distinct functions have different kinds of these, and possibly other, associated proteins. Studies of Mareck et al. (1980) showing changes in composition and activity of MAPs during brain development and a recent report by Izant et al. (1982).
MAPs. We show here, however, by immunofluorescence microscopy using antisera raised against individual MAP-1 and MAP-2 polypeptides, that in general only MAP-2 is missing from axons of rat brain, whereas MAP-1 is present, at least in myelinated axons. Microtubule-associated polypeptides immunoreactive with antiserum to MAP-1, were also identified in cultured neuroblastoma cells (clone N2A) by immunofluorescence microscopy as well as immunoautography of microtubule preparations polymerized from cell extracts.

Fig. 2. Immunofluorescence microscopy of frozen sections through rat cerebrum and cerebellum. (A), (C) and (E), antiserum to MAP-1. (B) and (D), antiserum to MAP-2. (F), antiserum to MAP-1 preabsorbed with purified high mol. wt. MAPs. (A) and (B), internal capsule of cerebrum. Note conspicuous immunostaining of axon-bundles (areas encircled by arrows) with antiserum to MAP-1 (A), but lack of staining of corresponding axons with antibodies to MAP-2 (B). (C)–(F), cerebellum. Axons present in the medulla (me) were stained by antibodies to MAP-1 (C), but not by antibodies to MAP-2 (D). Dendrites and bodies of nerve cells, Purkinje cells [arrows in (C) and (D)] included, in the molecular (m) as well as in the granular (g) layer of cerebellar cortex were stained by both antisera [(C), (D) and (E)]. The granular staining of Purkinje cells (arrow) seen in (F) is due to the non-immunospecific fluorescence of lipofuscin. Bars indicate 100 μm (A,B,C,D,F) and 40 μm (E).
separated by gel electrophoresis (Figure 1B), the antiserum raised against MAP-1 recognized antigenic determinants on both MAP-1 and MAP-2, whereas the antiserum raised against MAP-2 reacted almost exclusively with MAP-2. (A schematic section of the antiserum to MAP-2 with MAP-1...
Fig. 4. Immunofluorescence microscopy of N2A cells using antisera to MAP-1 and MAP-2. (A), (B), antisera to MAP-1, dilution 1/200. (C), antisera to MAP-2, dilution 1/50. Note continuous staining of microtubules in (A) and (B), but diffuse staining of the cytoplasm, predominantly around the nucleus in (C).

By immunofluorescence microscopy of cultured cells, we observed intensive staining of the neuroblastoma cell line N2A by antisera to MAP-1 (Figure 4A and B). Although in general the observation of structural detail is impeded by the spherical shape of this cell type, filamentous structures decorated by the antisera were clearly visualized in the cytoplasm of (sparse) well-spread cells (Figure 4A) as well as in cell extensions (Figure 4A and B). Similar structures were stained in N2A cells by antisera to microtubule protein (not shown). Staining of N2A cells was also observed with the antisera to MAP-2, though only at considerably lower serum dilutions (Figure 4C). Continuous filaments were, however, hardly visualized, but cells were stained diffusely primarily around their nuclei.

N2A cells, therefore, seemed to be a promising system for biochemically probing the association of MAP-1 with neuronal microtubules. Vallee (1982) recently reported the isolation of microtubules and microtubule-associated proteins from brain and HeLa cells by a taxol-dependent procedure. Microtubules polymerized from soluble extracts of N2A cells by a similar procedure contained a prominent polypeptide band co-migrating with hog brain MAP-1 upon SDS-polyacrylamide gel electrophoresis (Figure 5; lane 2). Using the antibody gel overlay technique, this band was shown to be specifically immunoreactive with antisera to MAP-1 but not with antisera to MAP-2 (Figure 5B).
Discussion

Our findings of MAP-1 in myelinated axons of neurons is in agreement with recent biochemical studies of Vallee (1982) showing an enrichment of MAP-1 in axon-rich white matter over gray matter of calf brain. Matus et al. (1981) recently showed immunostaining of dendrites and cell bodies but lack of staining in axons of neurons in various regions of rat brain using an antisera raised against fractionated preparations of high mol. wt. MAPs. These results would be partially contradictory to ours if the antisera applied had been reactive with both MAP-1 and MAP-2 as assumed by the authors (Matus et al., 1981). However, as suggested by Vallee (1982) and meanwhile reported by Matus et al. (First European Congress of Cell Biology, Paris, July 18–23, 1982) the antisera used in the original study (Matus et al., 1981) contained antibodies specific only for MAP-2 but not for MAP-1. This notion is further supported by the finding that our antisera specific for MAP-2 as well as another MAP-2-specific antisera (Miller et al., 1982) gave rise to staining patterns in rat cerebellum that were quite similar to those originally reported by Matus et al. (1981).

The compartmentalization of MAP-1 and MAP-2 in brain seems to be not merely a question of axonal versus dendritic location, because unmyelinated axons in the molecular layer of cerebellum apparently were non-reactive not only with antisera to MAP-1 but also with antisera to MAP-2. This raises the interesting possibility that microtubules of unmyelinated axons could be distinguishable from those of myelinated axons by their lack of MAP-1. However, for clarification of this point more extensive ultrastructural studies will have to be carried out.

Reports regarding the occurrence of high mol. wt. MAPs in cultured cells have been conflicting. While a number of laboratories reported their occurrence in various cell types including non-neuronal cells (Sherline and Schiavone, 1977; Conolly et al., 1978; Sheterline, 1978; Kuznetsov et al., 1980), other investigators restricted their distribution to cells of neuronal origin (Izant and McIntosh, 1980; Peloquin and Borisy, 1979). Since most of the data reported was based on immunological evidence it seems likely that differences in antibody specificities account for some of the conflicting results, especially as the specificities of some of the antisera applied were not fully established. Regarding neuroblastoma N2A cells, Izant and McIntosh (1980) reported no detectable antigen by immunofluorescence microscopy using a monoclonal antibody to hog brain MAP-2. This would be consistent with the finding reported here that N2A microtubules are preferentially associated with MAP-1. The relatively weak staining of N2A cells observed with our rabbit antisera to MAP-2, in principle could be due to the slight cross-reactivity of this antisera with MAP-1 (see above). However, this seems unlikely, because the staining pattern generated with this antisera differed from that observed with the antisera to MAP-2. Antibodies to MAP-1 clearly decorated continuous filamentous structures, even at the relatively high serum dilution of 1/200 (Figure 4A and B). Antibodies to MAP-2, however, gave rise to rather diffuse cytoplasmic staining, even when used at a lower (1/50) dilution (Figure 4C). Thus it appears that the antigens immunologically related to MAP-2 were located at sites different from those of MAP-1. In this context it should be noted that the titer of antibodies specific for MAP-2 is about twice as high in the antisera to MAP-2 as in the antisera to MAP-1 (H.Herrmann and G.Wiche, unpublished results). The notion of MAP-1 being the predominant high mol. wt. protein found in association with microtubules of N2A cells was fully supported by the in vitro polymerization experiments which showed that a polypeptide of the same size as hog brain MAP-1, and with cross-reactivity towards the antisemum to MAP-1, but not towards the antisemum to MAP-2, was a major component of microtubule proteins polymerized from soluble N2A cell extracts by taxol.

In conclusion, the data reported here suggest that, depending on the localization within different cell compartments, such as myelinated axons, non-myelinated axons or dendrites, neuronal microtubules are preferentially associated with distinct high mol. wt. proteins. Thus it is tempting to speculate that different complements of associated proteins determine distinct functions of neuronal microtubules. Cell lines of neuronal origin displaying microtubules affiliated with certain subsets of MAPs, such as the neuroblastoma N2A line, should be useful for the study of differential functions of MAPs and microtubules.

Materials and methods

**Immunization of rabbits**

Microtubule proteins were prepared from hog brain according to Shelanski et al. (1973). MAP-1 and MAP-2 were separated by preparative SDS-polyacrylamide gel electrophoresis. Protein bands were cut out from the gels after staining with Coomassie Blue and destaining in 7% acetic acid/10% isopropanol for several days. Bands were washed with 150 mM NaCl, 10 mM sodium phosphate, pH 7.0 (PBS) and homogenized with a glass-teflon homogenizer to yield a highly viscous suspension. Aliquots of this suspension were mixed 1:1 with Freund's adjuvant for the first or were used directly for later injections into rabbits. For the first injection, ~ 300 µg were used, for subsequent four boosters, 150 µg each were applied. Boosters were given at intervals of 2 weeks, except for the second and third injection. Animals were bled out 6 days after the final booster.

**Gel electrophoresis and immunautoradiography**

Electrophoresis in the presence of SDS was performed using the discontinuous buffer system of Laemmli (1970) and 7% polyacrylamide slab gels. For the detection of antigens on gels, the technique described by Burridge (1976) was used with several modifications (R.Pytela, Ph.D. Thesis, University of Vienna). Hog brain microtubule proteins prepared according to Shelanski et al. (1973), or taxol-polymerized neuroblastoma N2A cell microtubules, were separated by SDS-PAGE (7% polyacrylamide slab gels 0.8 mm thick), and bands were stained with 0.050% Coomassie Blue R 250 in 35% isopropanol/10% acetic acid for 10 min. After destaining in 7% acetic acid/10% isopropanol, lanes were incubated with antibody and separated and fixed overnight in 50% isopropanol/10% acetic acid. To remove residual stain, gels were washed with distilled water for 30 min and with 2 M sodium salicylate/10% isopropanol for 2–3 h. This was followed by equilibration of gels with 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.1% sodium azide (TBS) for 30 min, and TBS supplemented with 2.5% egg white for a further 30 min. To the latter solution, 25–150 µl of antiserum (depending on the size of the gel piece) were added, and incubation continued at 37°C with constant gentle shaking for 1–2 days. Excess of antiserum was washed away with TBS for 2–4 days, followed by incubation (1 day) with Staphylococcal protein A-trilatated (Tack et al., 1980) by reductive methylation (sp. act. ~ 1.5 x 10^6 c.p.m./µg; ~ 10^9 c.p.m. were used per incubation). Non-bound protein A was washed out with TBS for 2–4 days, gels were stained and destained as before, impregnated with 1 M sodium salicylate, dried onto filter paper and exposed for fluorography at ~ 70°C using Kodak XAR-5 film.

**Immunofluorescence microscopy**

Experimental details regarding immunofluorescence microscopy of tissue sections have been described previously (Franke et al., 1979). Sections through rat brain (4 µm) were stained with 1/50 dilutions of antisera in 140 mM NaCl, 2 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4 (PBS), followed by 1/15 dilutions of FITC-conjugated swine anti-rabbit-IgG (Dako-Immunoglobulins, Copenhagen) in PBS.

For immunofluorescence microscopy of neuroblastoma N2A cells, the cultures attached to glass coverslips were kept in Dulbecco's minimal essential medium without serum for 48 h prior to fixation to induce the outgrowth of...
cell extensions. Microscopy was then performed as previously described (Wiche and Baker, 1982), except that the treatment of cells with acetic acid was omitted. Primary antisera were used at dilutions indicated, secondary antibody preparations (FITC-conjugated goat anti-rabbit IgG; Nordic Laboratories) at dilutions of 1/20 in PBS.

**Immunoelectron microscopy**

Rat brain was fixed by perfusion of anesthetized animals through the left ventricle with 4% paraformaldehyde, 0.5% glutaraldehyde and 50 mM cacodylate buffer, essentially following procedures described previously (Matus et al., 1979). Sections (50 μm thick) prepared with a tissue chopper, were processed for immunoelectron microscopy using the peroxidase method (Sternberger et al., 1970) according to protocols given by Franke et al. (1981). Primary antisera as well as the peroxidase-conjugated anti-rabbit-IgG (Dako-Immunoglobulins, Copenhagen) used as secondary antibodies were applied at dilutions of 1/20 in PBS.

**Polymerization of microtubules from N2A cells**

Essentially the taxol method of Vallee (1982) was used. Cells grown in roller bottles were rinsed twice with PBS, harvested and suspended in 100 mM monosodium 1,4-piperazinediethane sulphonate, pH 6.9, 1 mM MgSO4, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 5 mM 2-mercaptoethanol, 0.1 mM GTP, 0.2 mg/ml N-tosyl-L-phenylalanylchloromethane (buffer A) at 1 g (wet weight)/ml. Cells were then sonicated and soluble extracts prepared by centrifugation at 180 000 g for 90 min at 4°C. Taxol and GTP were subsequently added to the extracts at final concentrations of 20 μM and 1 mM, respectively, and mixtures incubated for 30 min at 37°C. Microtubules formed were centrifuged through a cushion of 5% sucrose in buffer A, and redissolved for electrophoresis.

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**References**


