Colocalization of Microtubule-associated Protein 1A and Microtubule-associated Protein 2 on Neuronal Microtubules In Situ Revealed with Double-Label Immunoelectron Microscopy

Yoko Shiomura and Nobutaka Hirokawa

Department of Anatomy and Cell Biology, School of Medicine, University of Tokyo, Tokyo, Japan

Abstract. Microtubule-associated protein 1A (MAP1A) and microtubule-associated protein 2 (MAP2) were shown to be colocalized on the same microtubules (MTs) within neuronal cytoskeletons by double-label immunoelectron microscopy. To investigate the electron microscopic disposition of MAP1A and MAP2 and their relationship to MTs in vivo, and to determine whether there are different subsets of MTs which specifically bind either MAP1 or MAP2, we employed a double-label immunogold procedure on rat cerebella using mouse monoclonal antibody against rat brain MAP1A and affinity-purified rabbit polyclonal antibody against rat brain MAP2. MAP1A and MAP2 were identified with secondary antibodies coupled to 10- and 5-nm gold particles, respectively. In Purkinje cell dendrites, both 10- and 5-nm gold particles were observed to be studded on the fuzzy structures attached to the same MTs. Many such structures connected MTs to each other. There was no particular MT which bound either MAP1A or MAP2 alone. Furthermore, there seemed to be no specific regions on MTs where either MAP1A or MAP2 was specifically attached. Hence, we conclude that MAP1A and MAP2 are colocalized on MTs in dendrites and assume that MAP1A and MAP2 have some interrelationship in vivo and that their interactions are responsible for forming the network of cross-bridges between MTs and MTs in neuronal cytoskeletons.

Biochemical and immunological approaches have identified microtubule-associated protein 1 (MAP1),[1] microtubule-associated protein 2 (MAP2), and tau as the main MAPs in mammalian nerve cells. They have also revealed the specific localization of those MAPs in neurons. MAP1 occurs in equal amounts in both axons and dendrites (3, 6, 8). Although MAP2 is abundant in dendrites, it is present only in small amounts in axons (1, 8, 10). In contrast, tau is prominent in axons (2, 8). This biochemical differentiation of cytoskeletons is a characteristic feature of the cytological differentiation of neurons. To gain an understanding of this phenomenon, it is necessary to establish whether nerve cells contain segregated populations of microtubules (MT) that associate with specific MAPs.

The distributions of both MAP1 and MAP2 in nerve cells have as yet not been demonstrated by electron microscopy using a double-label procedure. The distribution of MAP1 in axons and dendrites at the molecular level was demonstrated in our previous studies by immunoelectron microscopy using the colloidal gold method (5, 9), which showed that MAP1 is a component of filamentous structures between microtubules in axons and dendrites (5, 9). MAP2 localization along dendritic MTs, on the other hand, was investigated using the peroxidase method (1).

1. *Abbreviations used in this paper*: MAP(s), microtubule-associated protein(s); MAP1A and MAP2, microtubule-associated proteins 1A and 2; MT(s), microtubule(s); pAb, polyclonal antibody.

We performed a double-label immunogold procedure on rat cerebella using anti-MAP1A monoclonal antibody (mAb) and anti MAP2 polyclonal antibodies (pAbs) in order to determine, by showing their localization at the molecular level, whether each MT in vivo associates with only MAP1 or MAP2, or associates with both of the MAPs.

Materials and Methods

Antibody Characterization

We prepared a monoclonal antibody (mAb) against rat brain MAP1A (9) and affinity-purified rabbit polyclonal antibodies (pAb) against rat brain MAP2 (kindly provided by Dr. Yasuo Ihara and Dr. Kei Mori, Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan). The pAb were affinity-purified using a column containing rat brain MAP2. Immunoreactive proteins from rat brain were identified by western blotting. Crude extracts and MT proteins isolated from rat brain by the taxol method (10) were fractionated on 7% SDS-polyacrylamide gels according to the method of Laemmli (7) and transferred to nitrocellulose paper. High molecular weight MAPs of brain MT proteins were further separated on 4% SDS/urea gels (3).

Double-Label Immunofluorescence Microscopy

Cerebella were dissected from rats that were perfused with 2% paraformaldehyde, 0.1% glutaraldehyde in PEM buffer, pH 6.8 (100 mM Pipes, 1 mM EGTA, 1 mM $MgCl_2$). After fixation for 2 h, the tissues were incubated for 30 min with 1% $NaBH_4$ in phosphate buffer containing 4% sucrose. After cryoprotection with a graded concentration series of sucrose solutions, frozen sections were prepared for immunohistochemistry.

Sections were incubated with primary antibodies to MAP1A and MAP2

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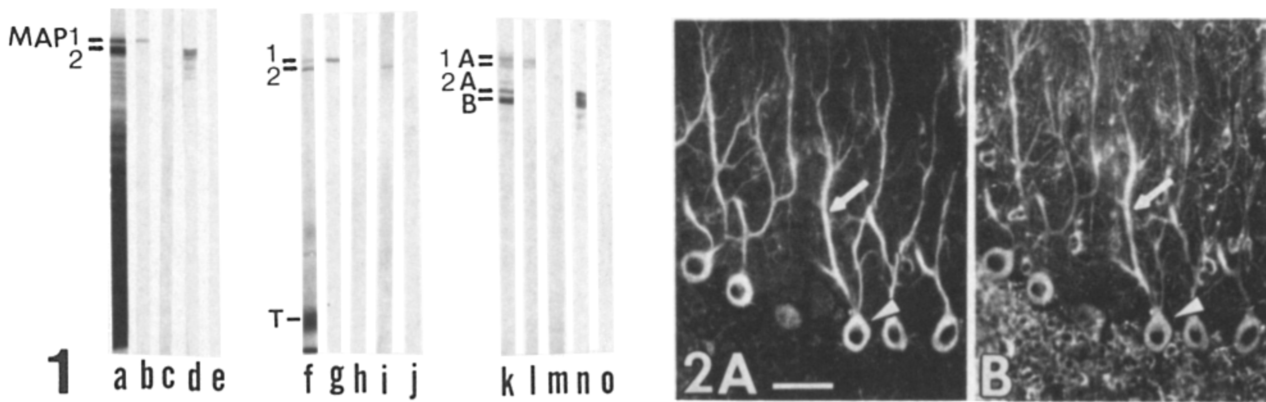


Figures 1 and 2. (Fig. 1) Characterization of mAb and pAb. Crude extracts (lane *a*) and microtubule proteins obtained from rat brain by the taxol method (10) (lane *f*) were fractionated on 7% SDS-polyacrylamide gels, and the specificities of antibodies were examined by Western blottings (lanes *b*, *c*, *d*, *e*, and *g*, *h*, *i*, *j*). mAb recognized MAP1 (lanes *b* and *g*), and conditioned medium of myeloma cells showed no staining (lanes *c* and *h*). Affinity-purified rabbit pAbs identified two bands of MAP2 strongly, with a few minor bands that were degraded MAP2 (lanes *d* and *i*). Normal rabbit serum was used as a control (lanes *e* and *j*). High molecular weight MAPs were further separated into their subclasses on 4% SDS/urea-gels (3) (lane *k*), and antibodies were examined on the blots (lanes *l*, *m*, *n*, and *o*). Our mAb against MAP1 reacted with two bands of MAP1A (l) as we stated previously (9), and pAb against MAP2 reacted with MAP2A and MAP2B (lane *n*). Lanes *m* and *o*, controls for each antibody, showed no reaction. T, tubulin. (Fig. 2) Double-labeled frozen section of rat cerebellar cortex stained with anti-MAP1A and anti-MAP2. MAP1A stained Purkinje cell bodies and dendrites strongly (*A*), and MAP2 reacted with identical Purkinje cell bodies and dendrites (*B*). (*Arrowheads*) Purkinje cell body; (*arrows*) Purkinje cell dendrite. Bar, 40 μm.

simultaneously, followed by an incubation with secondary antibodies containing rhodamine-conjugated goat anti-mouse IgG (Cappel Laboratories, Malvern, PA) and fluorescein-conjugated goat anti-rabbit IgG (Cappel Laboratories). As a control, sections were incubated with conditioned medium from myeloma cells and diluted nonimmune normal rabbit serum.

Double-Label Immunoelectron Microscopy

Rat cerebellum was processed for immunoelectron microscopy as described previously (9), except for solutions containing primary and secondary antibodies. Primary antibodies included either anti-MAP1A mAb and anti-MAP2 pAb, or conditioned medium from myeloma cells and normal rabbit serum (as a control); for secondary antibodies we used 10 nm gold-conjugated goat IgG anti-mouse IgG for mAb and 5 nm gold-conjugated goat IgG anti-rabbit IgG for pAb (Janssen Pharmaceutica, Beerse, Belgium).

Results and Discussion

Antibody Characterization

The mAb used in this study reacted strongly with MAP1 on 7% SDS-gel blots to which crude extracts and MT proteins of rat brain were transferred, and MAP1A was detected specifically on 4% SDS/urea-gel blots. The affinity-purified pAb recognized the bands of MAP2, with few minor bands of degradation products of MAP2, on every blot, while it did not cross-react with MAP1 (Fig. 1).

Double-Label Immunofluorescence Microscopy

Anti-MAP1A mAb stained Purkinje cell bodies, dendrites, and axons strongly (Fig. 2). In the identical section, MAP2 reacted with Purkinje cell dendrites strongly and with cell bodies variously, whereas it did not stain axons.

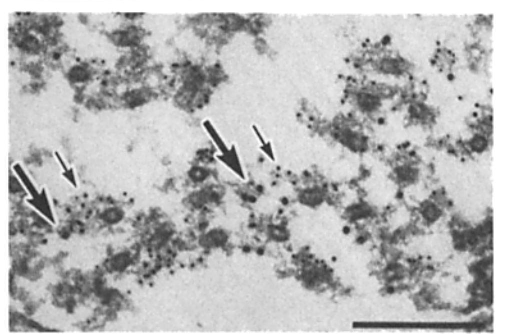
Double-Label Immunoelectron Microscopy

Fig. 3 shows a Purkinje cell dendrite double-labeled with anti-MAP1A and -MAP2 antibodies. Purkinje cell dendrites were identified by the presence of characteristic hypolemmal cisternae and postsynaptic thorns. Both 10- and 5-nm gold particles, which recognized MAP1A and MAP2, respectively, were specifically found within the dendrite. Both sizes of gold particles were associated with the same MTs. They were studded on the filamentous fuzzy materials that were attached to MTs, or connected MTs to adjacent MTs. Their labeling patterns seemed to be quite random. The inset shows clearly that MTs were attached by both MAPs. This cross-section view shows that gold particles were localized on the projecting portions of filamentous fuzzy materials between MTs, and that such fuzzy structures connected MTs to each other. Samples incubated with the control medium showed only slight nonspecific labeling (Fig. 4).

Our immunoelectron microscopic data showed that both MAP1A and MAP2 existed between MTs in dendrites, and that in most cases they connected MTs to each other. They were colocalized on MTs randomly; that is to say, there was no particular population of MTs which bound either MAP1A or MAP2 alone. In addition, there was no specific region on MTs where either MAP1A or MAP2 was specifically attached.

We previously demonstrated that the molecular structure of MAP1A was a long thin filamentous molecule with considerable flexibility (9). This structure was very similar to that of the MAP2 molecule (11). We also examined a three-dimensional ultrastructure of Purkinje cell dendrites by the

Figure 3. Electron microscopy of a double-labeled dendrite. Both 10- (*large arrows*) and 5-nm (*small arrows*) gold particles, which represented MAP1A and MAP2, respectively, were studded on the fuzzy materials existing along dendritic MTs specifically. MAP1A and MAP2 were distributed in a random fashion. Bar, 0.2 μm. (*Inset*) Cross section of a dendrite. Gold particles labeled projecting portions of the filamentous fuzzy materials between MTs. Each MT attached both MAP1A (10-nm gold particles, *large arrows*) and MAP2 (5-nm gold particles, *small arrows*). Pcd, Purkinje cell dendrite; Hc, hypolemmal cisternae; Plal, plasmalemma. Bar, 0.2 μm.



quick-freeze, deep-etch method (9). As a result, an extremely elaborate network of cross-bridges was demonstrated between MTs. The dendritic cross-bridges branching and anastomosing between MTs were more complex than cross-bridges observed in axons (4, 5). These findings, together with the immunofluorescence microscopic studies of MAP1A and MAP2 in dendrites (1, 3, 6, 8), the studies of the molecular structures of MAP1A and MAP2 (9, 11), and the quick-freeze, deep-etch studies of dendrites (9) and axons (4, 5), support our hypothesis that MAP1A and MAP2 may

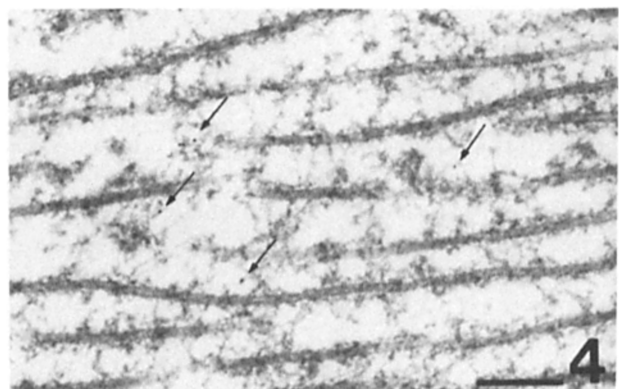


Figure 4. Control for double-label immunoelectron microscopy. Control showed slight nonspecific labeling. Arrows indicate gold particles as a result of nonspecific reactions. Bar, 0.2 μm.

have some interrelationship in vivo and that their interactions may result in the elaborate network of cross-bridges between MTs in dendritic cytoskeletons.

This kind of molecular dissection of neuronal cytoskeletons in situ is necessary for reaching an understanding not only of the cytological differentiation of nerve cells but also of the fundamental characteristics of MAPs.

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