Microtubule-associated Proteins of Neurons

MARK M. BLACK and JEFREY T. KURDYLA
Department of Anatomy, Temple University School of Medicine, Philadelphia, Pennsylvania 19140

ABSTRACT Microtubule-associated proteins (MAP) have been identified in cultures of rat sympathetic neurons. In all of the experiments performed here, the cultures consisted of >97% neurons. 26 proteins were identified in these neuronal cultures that (*a*) remained associated with cytoskeletons prepared with a Triton X-100-containing microtubule-stabilizing buffer, (*b*) were released from such cytoskeletons by incubation in microtubule-depolymerizing buffers, (c) were not detected in cytoskeletons prepared from cultures depleted of microtubules by treatment with podophyllotoxin, and (*d*) co-cycled with rat brain microtubule proteins. We conclude that these 26 proteins are associated with microtubules in sympathetic neurons. Two of these proteins have molecular weights of ~30,000 and isoelectric points of ~6.2; the rest of the proteins range in molecular weight from 60,000 to 76,000 and isoelectric point from 6.3 to 6.9. This latter group of MAPs was heat labile. Several other proteins in the neuronal cultures had the solubility properties and drug-lability expected of MAP. All of these proteins had apparent molecular weights >200,000; one of these putative MAP co-migrated with rat brain MAP-1. We did not detect any putative MAP in these cultures that co-migrated with rat brain MAP-2.

In isoelectric focusing–SDS PAGE, the 24 MAP with molecular weights of 60,000–76,000 appeared to comprise four distinct molecular weight classes. Each molecular weight class was in turn composed of several proteins that varied in isoelectric point. In peptide mapping experiments, the isoelectric variants of each molecular weight class gave rise to very similar peptide maps. These observations suggest that each molecular weight class consists of several closely related proteins. It was also determined that all except the most basic member of the four MAP classes could be phosphorylated in vivo, raising the possibility that differential phosphorylation contributed to the variation in the isoelectric points of the members of each MAP class.

We performed pulse-chase experiments to further evaluate the contribution of posttranslational modification to the generation of the complex population of MAP in the molecular weight range of 60,000 to 76,000. In cultures labeled for 20 min, only the more basic members of each MAP class were detectably labeled, while in cultures labeled for 20 min and then chased for 220 min the more acidic members of the MAP classes became labeled. The labeling of the more acidic MAP also occurred when the protein synthesis inhibitor emetine was included in the chase medium. These data suggest that for each MAP class its more acidic members are derived from one or more precursors by posttranslational mechanisms.

The distribution of the various microtuble proteins between assembled and unassembled states was examined. Triton X-100-soluble and -insoluble fractions, containing unassembled microtubule proteins and microtubules, respectively, were prepared from [^{35}S]methionine-labeled cultures and analyzed by isoelectric focusing–SDS PAGE and fluorography. With the extraction conditions used, the majority of the tubulin (74%, n = 4) fractionated with microtubules. Visual inspection of the fluorographs indicated that the MAP showed considerable variation in their partitioning between Triton X-100-soluble and -insoluble fractions, and quantitative analyses confirmed this impression. The fractionation of the MAP between Triton X-100-soluble and -insoluble fractions may reflect their in vivo distribution between unassembled and assembled states, respectively.

The axonal and dendritic cytoskeletons differ in composition and organization. Microtubules and neurofilaments are conspicuous elements of the axonal cytoskeleton. Neurofilaments are concentrated in the central region of the axon, and small clusters of microtubules are irregularly spaced within the neurofilament array (15, 30). The observation of filamentous

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elements that appear to cross-link neurofilaments with each other and with microtubules has led to the concept of a microtubule-neurofilament network in axons (22, 27). The existence of such a network has been confirmed by axonal transport studies that indicate that microtubules and neurofilaments are co-transported as components of a single structural complex (3). The dendritic cytoskeleton differs in several respects from the axonal cytoskeleton. For example, microtubules are the most prominent component of the dendritic cytoskeleton (25, 28). Neurofilaments are often absent from dendrites and, when present, are relatively minor components of dendritic cytoplasm (25). Second, microtubules have a relatively uniform distribution across dendrites (25, 28). Moreover, the packing density of microtubules in dendrites differs from that in axons. These differences in the cytoskeleton of axons and dendrites may contribute to the differences in their general shapes and also to the differences in their ability to modify their shapes in response to external stimuli.

The present study is part of a long-term project to define the cellular basis for producing and maintaining the differing patterns of microtubule organization in axons and dendrites. Microtubules are complex polymers, consisting of tubulin and one or more classes of microtubule-associated proteins (MAP)[1] (17). MAP appear to influence the spatial pattern of microtubules in cells (16) and they may also influence the interactions of microtubules with other cytoplasmic structures (13, 20, 29, 31). Thus, it is possible that differences in the composition of MAP may contribute to the differences in microtubule organization in axons and dendrites.

Studies to date have identified three classes of MAP in soluble extracts of brain, one of high molecular weight (HMW) (23, 32) and two of comparatively lower molecular weight designated tau (8) and low molecular weight (LMW) MAP (1, 35). The initial identification of these proteins as MAP was based on their ability to copurify with tubulin through several assembly-disassembly cycles. More recently, proteins antigenically related to tau and HMW MAP have been detected in a number of tissue culture cells, in which their distribution closely resembles that of microtubules (9, 11). Proteins antigenically related to tau and HMW MAP have also been detected in neurons (9, 21). Several observations suggest that these proteins are not uniformly distributed between axons and dendrites. For example, at least one of the HMW MAP appears to be enriched in dendrites relative to axons (21, 35). Also, only a subset of the total brain tau class of MAP has been detected in axons (3, 34), raising the possibility of differential localization of these proteins in neurons. The apparent selective partitioning of MAP between axons and dendrites may contribute to the differences in microtubule organization in these processes. To further evaluate this relationship, it is first necessary to rigorously identify the MAP of neurons and then to determine their precise localization within the neuron. The purpose of the present experiments is to characterize the MAP of neurons. For these experiments, we used pure neuronal cultures as starting material to identify neuronal MAP. The relatively stringent criteria established by Solomon et al. (33) were used to identify MAPs in these cultures. With these criteria, we have identified a large number of proteins in cultured neurons that have several properties expected of MAP. A preliminary account of this work has been published (4).

[1] *Abbreviations used in this paper:* 1-D, one-dimensional; 2-D, two-dimensional; HMW, high molecular weight; LMW, low molecular weight; MAP, microtubule-associated protein.

MATERIALS AND METHODS

Neuronal Cultures: Superior cervical ganglia were dissected from 1–5-d-old rat pups, incubated in 0.25% collagenase (Worthington Biochemical Corp., Freehold, NJ) for 60 min (at 37°C) followed by 0.25% trypsin (Worthington) for 40–50 min (at 37°C), and then dissociated by trituration in medium (see below) to obtain a single cell suspension. Dissociated cells were plated onto Costar culture dishes coated with rat-tail collagen. Cultures were grown either in RPMI 1640 as described previously (2) or in Eagle's MEM adjusted to 50 U/ml penicillin, 50 mcg/ml streptomycin, 0.02 M KCl, 0.7% wt/vol glucose, 2% vol/vol chick embryo extract, and 10% fetal calf serum. All media contained nerve growth factor at 50 ng/ml. The profile of MAP identified in the cultures was not detectably affected by the different media. Nonneuronal cells were eliminated from the cultures by treatment with 10 μM cytosine arabinoside. Treatment was maintained until nonneuronal cells were no longer observed upon microscopic examination (usually 5–7 d). Cultures were fed daily or every other day and, unless otherwise indicated, were maintained for 14–21 d before use.

Metabolic Labeling: For most experiments, cultures were fed daily with medium containing 15–20% of the normal amount of methionine plus 50–100 μCi/ml of [^{35}S]methionine (New England Nuclear, Boston, MA) for the 2–3-d period immediately before extraction (see below). To determine whether the MAP were phosphoproteins, cultures were incubated with 1–2 mCi [^{32}P]-PO_4 (obtained from ICN Pharmaceuticals, Inc., Irvine, CA) in phosphate-free medium for 2 h before extraction.

Detergent Extraction: Cultures were extracted with Triton X-100 under solution conditions that preserved existing microtubules and allowed quantitative separation of polymerized and unpolymerized tubulin (see reference 2 for details). Most experiments employed the homogenization extraction procedure described previously (2). Briefly, cultures were rinsed twice with Ca^{++}-Mg^{++}-free phosphate-buffered saline (PBS), once with a microtubule-stabilizing buffer (consisting of 0.1 M PIPES, pH 6.9, 4 M glycerol, 5% vol/vol dimethylsulfoxide, 1 mM $MgSO_4$, 1 mM EGTA, and approtinin [0.02 trypsin inhibitory units per ml]), and then scraped into microtubule-stabilizing buffer + 0.5% wt/vol Triton X-100 and homogenized with a Dounce homogenizer. The homogenate was centrifuged at 33,000 rpm in the Beckman type 65 rotor (Beckman Instruments, Inc., Palo Alto, CA) for 45 min at room temperature. Unpolymerized microtubule proteins are soluble in the detergent-containing microtubule-stabilizing buffer and remain in the supernate, while polymerized microtubule proteins are insoluble and therefore pellet. After gentle rinsing of the pellet surface with microtubule-stabilizing buffer without EGTA, the pellet was resuspended in cold (4°C) 0.1 M PIPES, pH 6.9, 1 mM $MgSO_4$, 1 mM EGTA, and approtinin, and incubated for 30 min on ice. This cold incubation depolymerizes the microtubules in the Triton X-100-insoluble pellet, and thereby solubilizes their component proteins. The resulting suspension was clarified by centrifugation (12,000 *g* for 10 min at 4°C) yielding a cold-soluble fraction that contained ~92% of the tubulin in the Triton X-100-insoluble pellet (2).

For some experiments, neurons were extracted while remaining attached to the dish (dish extraction procedure [2]). The extraction protocol involved rinsing the cultures twice with PBS, once with buffer 1 (2 M glycerol, 0.1 M PIPES, pH 6.9, 1 mM $MgSO_4$, 2 mM EGTA, approtinin), and then incubating the culture for two successive 10-min periods with buffer 2 (buffer 1 + 0.2% wt/vol Triton X-100). The resulting Triton X-100-insoluble residues, which contained the stabilized microtubules (2), were rinsed twice with buffer 3 (buffer 1 without EGTA) and then scraped into the Ca^{++} buffer (0.1 M PIPES, pH 6.9, 1 mM $MgSO_4$, 5 mM $CaCl_2$, approtinin), incubated at 4°C for 10 min, and then clarified by centrifugation (12,000 *g* for 10 min at 4°C) to yield a Ca^{++}-soluble fraction containing the products of microtubule depolymerization.

Cold-soluble or Ca^{++}-soluble fractions prepared as described above were obtained from control cultures and from cultures depleted of microtubules by incubation with 5 μg/ml podophyllotoxin for the 18–24 h immediately before extraction. Triton X-100-insoluble residues from control cultures contained microtubules, while those from drug-treated cultures did not. Thus, the cold- or Ca^{++}-soluble fractions from control cultures will contain microtubule proteins while the cold- or Ca^{++}-soluble material from drug-treated cultures will not. In the initial identification of neuronal MAP, labeled proteins in cold- or Ca^{++}-soluble fractions prepared from control and drug-treated cultures were visualized by isoelectric focusing–SDS polyacrylamide gel electrophoresis (two-dimensional [2-D] PAGE) and fluorography. Proteins present in cold- or Ca^{++}-soluble fractions from control cultures, but absent from cold or Ca^{++}-soluble fractions from podophyllotoxin-treated cultures, were candidates for MAP.

Co-assembly of Neuronal MAP with Brain Microtubule Proteins: Cold-soluble fractions prepared from [^{35}S]methionine-labeled control cultures were prepared as described above and mixed with an equal volume of 20 mg/ml twice-cycled microtubule proteins prepared from rat brain. Rat brain microtubule proteins were prepared as described by Herzog and Weber (14). The mixture of rat brain microtubule proteins and the labeled proteins of the cold-soluble fractions was then carried through two or three cycles of temperature-dependent assembly-disassembly (in the absence of glycerol), and the final assembly pellet was depolymerized at 4°C, clarified by centrifugation, and then analyzed by 2-D PAGE and fluorography.

Analytic Procedures PAGE: One-dimensional (1-D) PAGE was performed according to reference 18. 2-D PAGE was carried out as previously described (2). The pH gradients obtained ranged from 4.5 to 7.3. The second dimension consisted of 7.5% acrylamide. Labeled proteins in the 1-D and 2-D gels were visualized by autoradiography or fluorography (5).

Peptide Mapping: Peptide mapping following limited proteolysis (7) was performed using 150 ng *S. aueurus* V. 8 protease (Miles Laboratories, Elkhart, IN). Samples for peptide mapping were excised from 2-D gels. The proteolytic fragments generated by the protease were separated on gels consisting of a 10–20% gradient of acrylamide and then visualized by fluorography.

RESULTS

Neurons Have a Complex Population of MAP

In the experiments reported here, pure neuronal cultures were extracted with Triton X-100 under solution conditions that stabilized most or all of the existing microtubules in the cultures. With these extraction conditions, the stabilized microtubules remained insoluble, while unpolymerized microtubule proteins were quantitatively solubilized (2). Triton X-100-insoluble fractions were prepared from control cultures and cultures depleted of microtubules by treatment with 5 μg/ml podophyllotoxin. The resulting Triton X-100-insoluble fractions were then incubated in a microtubule-depolymerizing buffer at 4°C. We have previously shown (2) that this treatment solubilizes ~92% of the proteins associated with microtubules, as well as extraneous proteins (some of these latter proteins may also be microtubule proteins [see Discussion and reference 33]). Cold- or Ca^{++}-soluble fractions obtained from control cultures will contain microtubule proteins plus the extraneous proteins, while similarly prepared fractions from drug-treated cultures will contain only the extraneous proteins. Thus, peptides present in cold- or Ca^{++}-soluble fractions prepared from control cultures, but absent from the fractions prepared from drug-treated cultures, are candidates for MAP (see references 12, 24, and 33 for detailed discussions of the methodology).

Fig. 1, *a* and *b* depict fluorographs of 2-D gels of cold-soluble fractions obtained from control and drug-treated cultures, respectively. 26 spots were identified in the fluorographs of control samples that were absent or barely detectable in the fluorographs of drug-treated samples (arrowheads). Two of these spots correspond to proteins with apparent molecular weights of ~30,000 and isoelectric points of ~6.2; the remaining spots correspond to proteins which range in apparent molecular weights from 60,000 to 76,000 and isoelectric points from 6.3 to 6.9. We tentatively identify these polypeptides as neuronal MAP. The same 26 proteins were also identified as candidates for MAP in cultures extracted according to the dish-extraction procedure (see Materials and Methods).

If the proteins identified above are MAP, then they should co-cycle with brain microtubule proteins. To test this prediction, cold-soluble fractions prepared from [^{35}S]methionine-labeled control cultures were mixed with unlabeled brain microtubule proteins and then carried through two or three cycles of temperature-dependent assembly-disassembly (see Materials and Methods). All of the proteins identified in Fig. 1 *a* as putative MAP co-cycled with brain microtubule proteins (Fig. 1 *c*, arrowheads). This observation was made in three separate experiments. Therefore, we conclude that the proteins identified in Fig. 1 *a* as putative MAP are MAP of rat sympathetic neurons. The relative abundance of the MAP before and after cycling varied somewhat from experiment to experiment. The data shown in Fig. 1 *c* are representative of

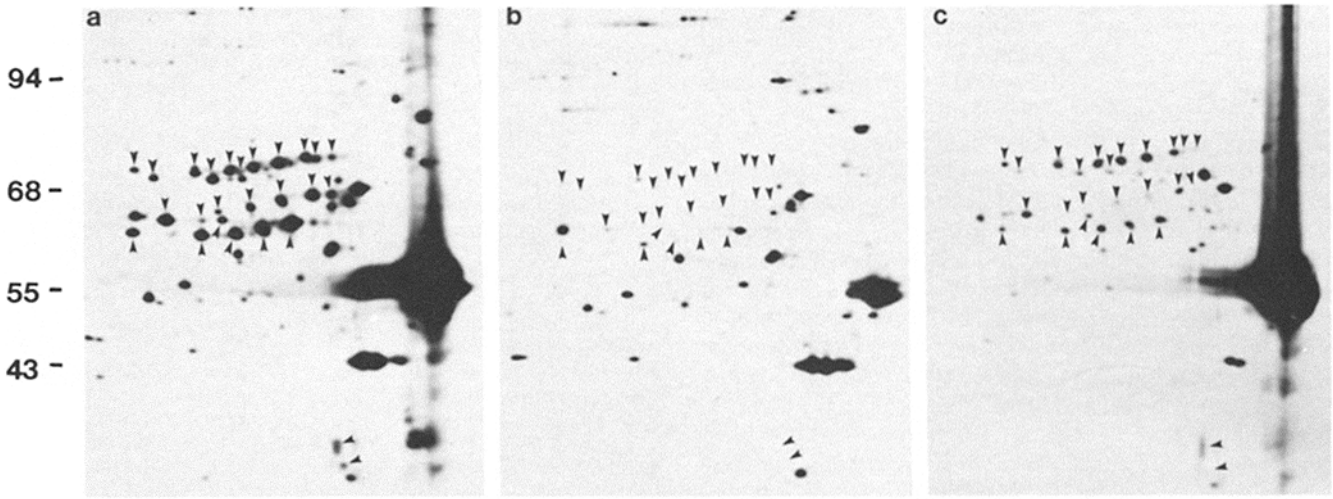

FIGURE 1 Fluorographs of portions of 2-D gels revealing the pattern of labeled proteins in the cold-soluble fraction prepared from (a) a control culture, or (*b*) a culture treated with 5 μg/ml podophyllotoxin for 24 h before extraction. The arrowheads indicate the proteins present in cold-soluble fractions from control cultures but absent from similarly prepared fractions from drug-treated cultures. These proteins are the putative MAP of neurons (see text). In a separate experiment, cold-soluble fractions prepared from radiolabeled control cultures were mixed with unlabeled rat brain microtubule proteins and carried through three assembly-disassembly cycles (see Materials and Methods). The final microtubule pellet was depolymerized at 4°C, clarified by centrifugation, and then analyzed by 1-D PAGE and fluorography. A portion of such a fluorograph is shown in c. The putative MAP are indicated by the arrowheads. Note that the proteins identified in panel a as putative MAP also co-cycled with brain microtubule proteins. Molecular weights at the left designated × 10^{-3}.

two of the three experiments. Note that the abundance of the middle series of spots (apparent molecular weights of 62,000–67,000) relative to the other spots appeared less in the cycled sample compared to the starting material. However, in a third experiment, these proteins appeared to co-cycle to the same extent as the other MAP. The reason for this variability is unknown.

One of the major classes of MAP in brain microtubule protein preparations has a HMW (>250,000), and consists of two distinct species, MAP-1 and MAP-2 (23, 32). We have examined cultured sympathetic neurons for MAP that correspond to brain HMW MAP. For these analyses, Ca^{++}- or cold-soluble fractions from control and drug-treated cultures were examined by 1-D PAGE rather than 2-D PAGE because the HMW MAP do not focus into discrete spots in 2-D PAGE. As seen in Fig. 2, several bands corresponding to proteins with relatively high molecular weights were present in fractions prepared from control cultures but absent from similarly prepared fractions from drug-treated cultures. One of these putative MAP was very similar in mobility to rat brain MAP-1 and another had a molecular weight of ~240,000 and migrated distinctly faster than rat brain MAP-2. The remaining two or three bands were closely spaced, and had apparent molecular weights slightly >200,000. These latter proteins may be analogous to the relatively HMW MAP present in many rodent cells (12). We have not determined whether these putative MAP will co-cycle with brain microtubule proteins. We did not detect putative MAP in these cultures that co-migrated with rat brain MAP-2.

The neuronal MAP with molecular weights ranging from 60,000 to 76,000 resemble the tau class of brain MAP in some respects (see Discussion). Brain tau has the unusual property of remaining soluble after incubation in a boiling water bath (8). To further evaluate the tau-like nature of these neuronal MAP, we have examined their solubility following incubation in a boiling water bath. Cold-soluble fractions prepared from control cultures were adjusted to 0.75 M NaCl and 2 mM 2-mercaptoethanol, and then incubated in a boiling water bath for 5 min. The boiled material was centrifuged for 10 min at 12,000 *g* and the resulting supernate and pellet were analyzed by 2-D PAGE (Fig. 3). Under these conditions, the neuronal MAP with molecular weights ranging from 60,000 to 76,000 were completely insoluble. Mixing the cold-soluble fraction with carrier brain microtubule proteins (at a final concentration of 5 mg/ml) did not alter the heat lability of the neuronal MAP. The MAP with molecular weights of ~30,000 were also insoluble after boiling.

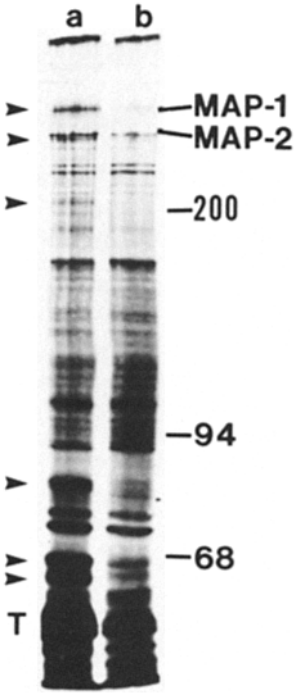

FIGURE 2 Autoradiograph of a 1-D gel (6% acrylamide) depicting the labeled polypeptides in Ca^{++}-soluble fractions prepared from control (*a*) and podophyllotoxin-treated (*b*) cultures. Unlabeled rat brain microtubule proteins were mixed with the labeled samples before electrophoresis to provide internal markers for MAP-1 and MAP-2. The arrowheads on the left identify labeled bands present in the control sample but absent from the drug-treated sample; *T*, tubulin. Molecular weights at right, $\times 10^{-3}$.

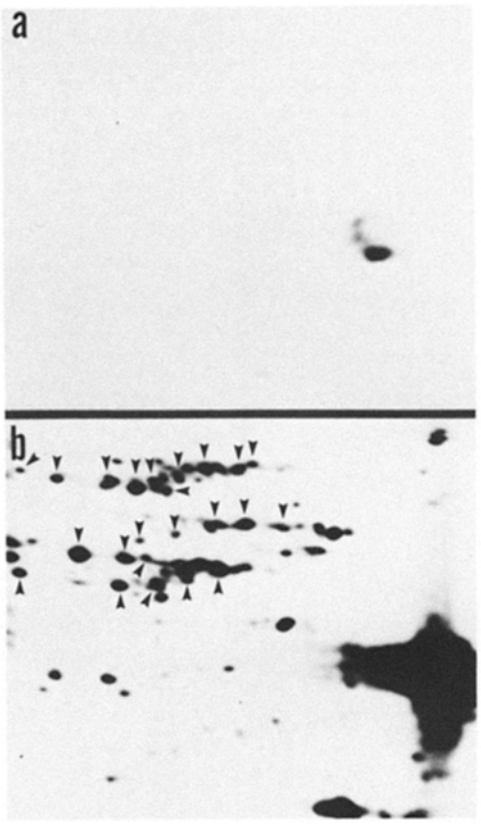

FIGURE 3 Portions of fluorographs of 2-D gels showing the labeled polypeptides present in the heat-soluble (*a*) and heat-insoluble (*b*) fractions prepared from cold-soluble material from a 14-d-old culture. The cold-soluble fraction was adjusted to 0.75 M NaCl and 2 mM 2-mercaptoethanol, incubated in boiling water bath for 5 min and then separated into soluble and insoluble material by centrifugation (12,000 *g* for 10 min at 4°C). The entire supernate and pellet were analyzed by 2-D PAGE. The fluorographs were exposed for 7 d.

24 MAP in the Molecular Weight Range of 60,000–76,000 Comprise Four Sets of Structurally Related Proteins

In 2-D PAGE, the MAP with apparent molecular weights ranging from 60,000 to 76,000 are organized into a series of approximately horizontally aligned spots that vary in isoelectric point. This organization raises the possibility that each horizontal series may consist of closely related proteins. To test this possibility, cold-soluble fractions from control cultures were analyzed by 2-D PAGE, and the spots corresponding to the various MAP were excised from the gels and peptide-mapped following limited proteolysis. Fig. 4*A* shows a portion of an autoradiograph of a 2-D gel showing the MAP of the cold-soluble fraction. The proteins analyzed by peptide

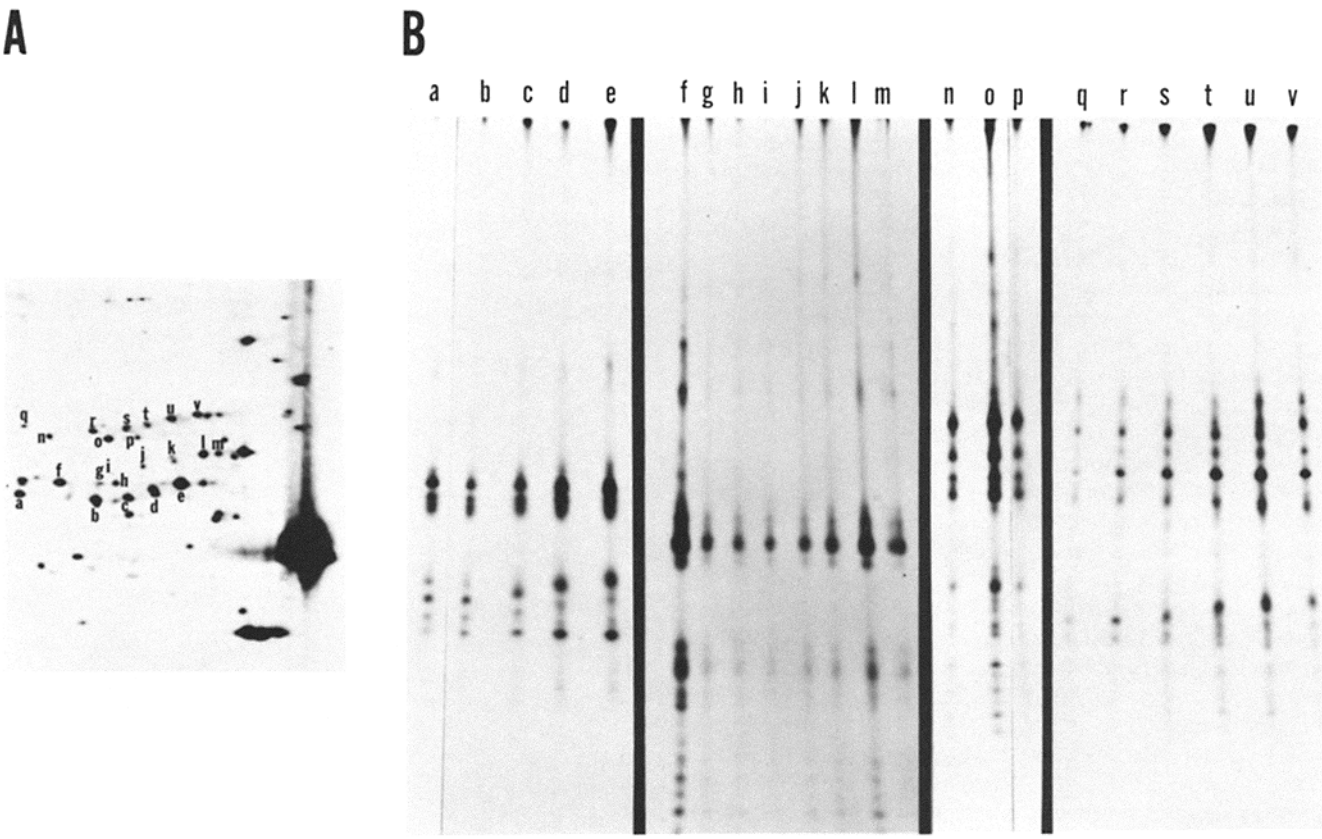

FIGURE 4 Several of the MAP in the molecular weight range of 60,000–76,000 were analyzed by 1-D peptide mapping. Cold-soluble fractions prepared from control cultures were subjected to 2-D PAGE and autoradiography, and the spots corresponding to the MAP were excised and peptide-mapped (see Materials and Methods). *A* shows a portion of an autoradiograph which depicts the MAP present in a typical cold-soluble fraction. The MAP analyzed by peptide mapping have been given a letter designation. *B* shows representative 1-D peptide maps of these proteins.

mapping have been given a letter designation. The proteins of the lowest horizontal row (designated *a*, *b*, *c*, *d*, and *e*, and collectively referred to as set *I*) gave rise to very similar peptide maps Fig. 4*B*). Similarly, the peptide maps of the eight proteins of the middle row (designated, *f*, *g*, *h*, *i*, *j*, *k*, *l*, and *m* [set *II*]) were also very similar to each other (Fig. 4*B*). The MAP of the upper row were divisible into two sets based on their peptide maps. One set (set *III*) consisted of at least three proteins (designated *n*, *o*, and *p*) while the other (set *IV*) consisted of at least six proteins (designated *q* through *v*). The proteins of set III gave rise to peptide maps that were indistinguishable from one another, and, similarly, the proteins of set IV had very similar peptide maps (Fig. 4*B*). However, peptide maps of the members of set III were distinct from those of set IV (not shown). These data indicate that the 24 MAP of cultured neurons with apparent molecular weights ranging from 60,000 to 76,000 can be divided into four distinct sets that differ in molecular weight. Each set consists of several proteins that vary in isoelectric point and, to a lesser degree, apparent molecular weight, but that give rise to very similar or indistinguishable peptide maps. These data are consistent with the possibility that each set consists of several closely related proteins, or variants of a common protein.

Neuronal MAP Are Phosphoproteins

It is well documented that MAP of brain and cultured cells can be phosphorylated in situ (24, 32), and that such phosphorylation generates isoelectric variants that can be resolved by 2-D PAGE (24). Thus, it is possible that phosphorylation contributes to the variation in isoelectric points of the MAP comprising each of the sets described above. To begin testing this possibility, we have determined whether the MAP in cultured sympathetic neurons are phosphoproteins. Control and podophyllotoxin-treated cultures were incubated with [^{32}P]PO_4 and then extracted, as described in Materials and Methods, to yield cold-soluble fractions. Analysis of these fractions by 2-D PAGE and autoradiography (Fig. 5*c* and *d*) revealed several phosphoproteins that behaved like microtubule proteins in that they were present in control samples but absent or diminished in drug-treated samples. In these experiments, the control samples contained sufficient protein to visualize tubulin and the MAP by Coomassie Blue staining. Comparison of the stained pattern of proteins with the pattern of phosphoproteins revealed that the major phosphoprotein that was present in control samples but absent in drug-treated samples co-migrated with tubulin (Fig. 5, *c* and *d*). Also, all except the most basic member of the four MAP sets were phosphorylated in situ. In addition, two MAP-like proteins were consistently detected in experiments using [^{32}P]PO_4 (←) but not [^{35}S]methionine. These proteins appear to be members of MAP set III.

Pulse-chase Experiments

The peptide mapping and phosphorylation data raise the possibility that the members of each MAP set are related as posttranslational variants of a common protein. To further

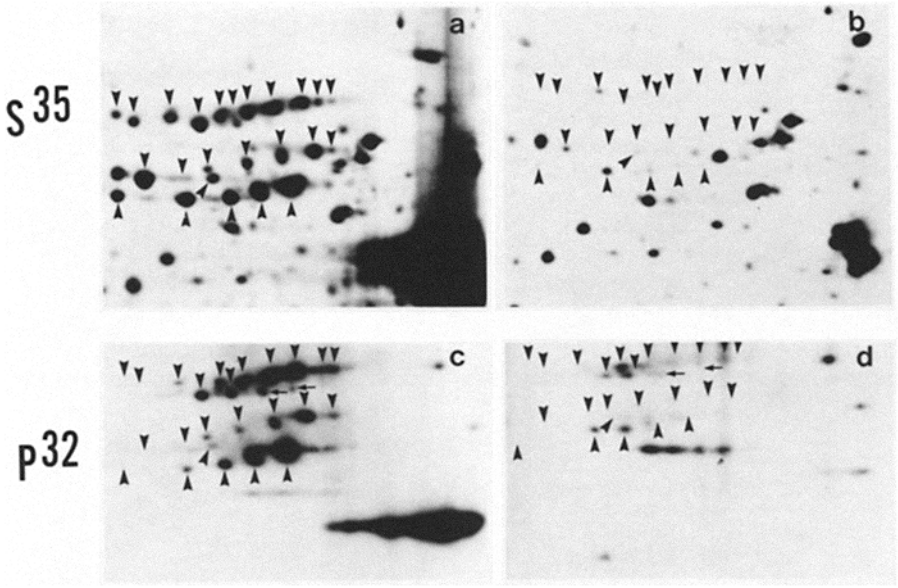

FIGURE 5 Autoradiographs of portions of 2-D gels of cold-soluble fractions prepared from [^{35}S]methionine-labeled cultures (*a* and *b*) or [^{32}P]PO_4-labeled cultures (*c* and *d*). *a* and *c* show the labeled proteins in cold-soluble fractions from control cultures, while *b* and *d* show the labeled material from similarly prepared fractions obtained from podophyllotoxin-treated cultures. The arrowheads identify the proteins present in the material from control cultures but absent or greatly diminished from drug-treated cultures. In *c* and *d*, the arrows indicate phosphoproteins that behaved like MAP but were not detected in experiments with [^{35}S]methionine.

test this possibility, we examined the patterns of labeled MAP obtained from cultures pulse-labeled for 20 min and then chased for various times between 0 and 220 min. In some experiments, the protein synthesis inhibitor emetine was included in the chase medium at 0.1 mM. This level of emetine inhibited the ability of the cultures to incorporate amino acids into proteins by ≥98%. After the designated chase time, cultures were extracted by homogenization to yield detergent and cold-soluble fractions. To visualize the labeling patterns of total MAP (assembled and unassembled), 10% of the detergent and cold-soluble fractions were mixed and then analyzed by 2-D PAGE and fluorography. The profiles of labeled MAP assembled into microtubules were revealed by examining the cold-soluble fractions.

The labeling patterns of total MAP that resulted from these pulse-chase experiments are illustrated in Fig. 6, *a–c*. When cultures were processed immediately after a 20-min pulse, only the more basic members of MAP sets I, II, and IV were detectably labeled (Fig. 6*a*). However, when labeled cultures were chased for 220 min before extraction, the more acidic members of these MAP sets became labeled (Fig. 6*b*); including emetine in the chase medium did not prevent the appearance of label in the acidic MAP (Fig. 6*c*). The labeling patterns of the members of MAP set III in these pulse-chase experiments differed from those of the other MAP sets in that all of the MAP of set III were labeled after a 20-min pulse, and their labeling pattern did not appear to change during the chase period. In other pulse-chase experiments, we were unable to chase [^{35}S]methionine entirely out of the more basic MAP and into the more acidic MAP of their respective MAP sets, even with chase times of 16 h. Instead, the labeling pattern of the total MAP from cultures chased for 16 h resembled the pattern observed in Fig. 6*b*.

The labeling patterns of the assembled MAP in these pulse-chase experiments mirrored those of total MAP. In cold-soluble fractions prepared from cultures labeled for 20 min (Fig. 6*d*), only the one or two most basic members of MAP sets I, II, and IV were detectably labeled (as with total MAP, all of the assembled members of set III were labeled). When labeled cultures were chased in the absence of [^{35}S]methionine, the more acidic members of these MAP sets gradually became labeled (Fig. 6, *e* and *f*), such that the labeling pattern of MAP in cultures chased for 220 min closely resembled that seen in cultures labeled continuously for 2–3 d. These changes in the labeling patterns of the assembled MAPs also occurred when emetine was included in the chase medium (data not shown). The results of these pulse-chase experiments suggest that the more acidic members of MAP sets I, II, and IV are derived from one or more precursors by posttranslational modification.

Solubility Properties of Neuronal MAP

Comparison of the labeling patterns of total and assembled MAP (see Fig. 6, *b* and *f*) suggested that certain MAP fractionate preferentially with microtubules. To further evaluate this possibility, an 8-d-old culture labeled with [^{35}S]methionine was dish-extracted to yield Triton X-100-soluble and Ca^{++}-soluble fractions, containing the microtubule proteins that were unassembled or assembled, respectively, at the time of cell extraction. The proteins of each fraction were analyzed by 2-D PAGE and fluorography. Fig. 7 illustrates representative fluorographs from such an experiment. The majority of the labeled tubulin fractionated with microtubules (compare Fig. 7*a* and *b*, large arrowheads). Visual inspection of the fluorographs revealed that the MAP (small arrowheads) showed considerable variation in their relative distribution between detergent-soluble and Ca^{++}-soluble fractions. Similar observations were also obtained with the homogenization extraction procedure (data not shown). Quantitative analysis of the distribution of the members of MAP set I (labeled *a*, *b*, *c*, *d*, and *e* in Fig. 4) between detergent-soluble and Ca^{++}-soluble fractions confirmed the impression derived from the fluorographs (see Table I). In particular, 65% of the most acidic member of this set (*e* in Table I) was present in the Ca^{++}-soluble fraction, while only 15% of the second member of this set (*b* in Table I) was present in this fraction.

DISCUSSION

MAP of Cultured Sympathetic Neurons

The present studies were aimed at identifying nontubulin proteins associated with neuronal microtubules in situ. For these studies, pure populations of neurons grown in tissue culture were used as the starting material for identifying these

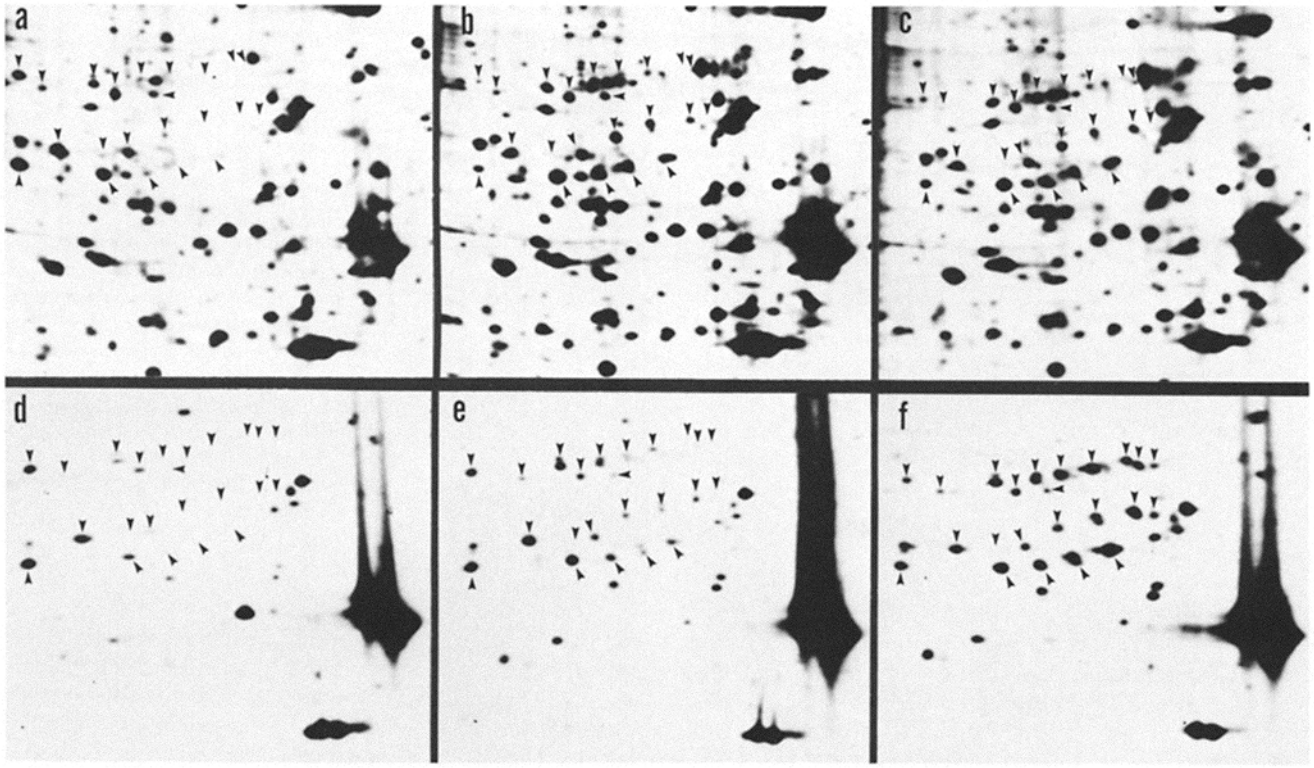

FIGURE 6 Fluorographs of 2-D gels showing the pattern of labeled polypeptides in fractions prepared from cultures pulse-labeled for 20 min and then chased for times ranging from 0 to 220 min. After the designated pulse-chase condition, cultures were extracted by homogenization to obtain detergent and cold-soluble fractions (see Materials and Methods). To visualize the labeling patterns of total MAP (i.e., assembled + disassembled MAP), 10% of the detergent and cold-soluble fractions from each culture were mixed prior to analysis by 2-D PAGE and fluorography. The remainder of the cold-soluble fraction was also analyzed to determine the labeling patterns of the assembled MAP. The labeling patterns of total MAPs from cultures chased for 0, 220, or 220 min with emetine (see text for details) can be seen in *a*, *b*, and *c*, respectively. The labeling patterns of the assembled MAP of cultures chased for 0, 140, or 220 min can be seen in *d*, *e*, and *f*, respectively. The MAP are indicated by arrowheads.

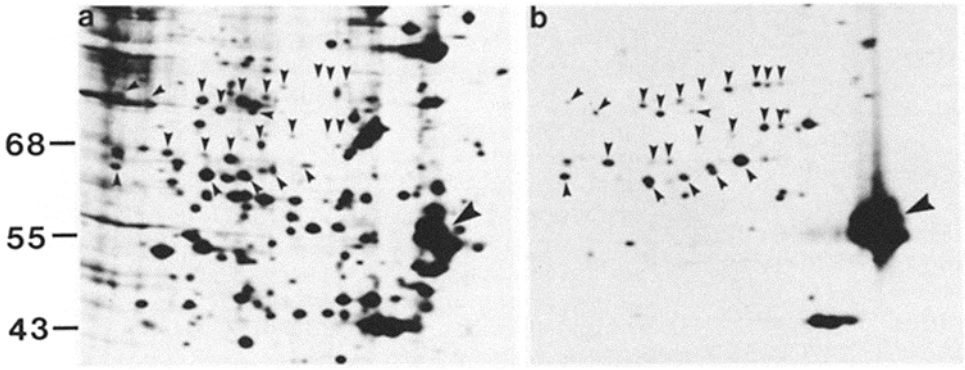

FIGURE 7 Fluorographs of portions of 2-D gels illustrating the labeled proteins in Triton X-100-soluble (*a*) and Ca^{++}-soluble (*b*) fractions prepared from an 8-d culture that was dish-extracted as described in Materials and Methods. The entire Triton X-100-soluble and Ca^{++}-soluble fractions were applied to the gels, and the gels were exposed to the film for 32 h. In *b*, the arrowheads identify the MAP with apparent molecular weights ranging from 60,000 to 76,000. In *a*, the arrowheads identify the proteins that co-migrate with these MAP.

proteins. This culture system has several advantages over brain for identifying neuronal MAP. First, with the culture system, the neuronal origin of the proteins identified is unambiguous. In contrast, identifying the cellular origin of brain MAP is complicated by the cellular heterogeneity of brain. Second, ~95% of the tubulin and presumably other microtubule proteins in the neuronal cultures can be solubilized under relatively gentle, nondenaturing conditions, thereby facilitating the identification of neuronal MAP. In contrast, the preparation of microtubule proteins from brain necessitates considerably harsher procedures to solubilize these proteins, and, even with these procedures, 50% or more of the brain tubulin and other microtubule proteins remain insoluble. Thus, studies with brain may identify only a portion of the microtubule proteins of this tissue, and these proteins may not be representative of all cell types or cellular domains (in this regard, see reference 6). Finally, MAP from brain are assayed primarily by their ability to co-cycle with tubulin under in vitro conditions (17). However, it has been demonstrated that co-cycling, in and of itself, is not a very stringent criterion for identifying a protein as a MAP (19). With the culture system, criteria in addition to co-cycling can be used

TABLE I

Distribution of Microtubule Proteins between Assembled and Unassembled Fractions

Tubulin	MAP a	b	c	d	e
74 ± 5	31 ± 6	15 ± 4	22 ± 6	33 ± 8	65 ± 6

To determine the distribution of tubulin and the MAP between Triton-soluble (unassembled) and Ca^{++}-soluble (assembled) fractions, the autoradiographs or fluorographs were used to identify the positions of these proteins in the gels. The appropriate region of the gels were then excised, dissolved in 30% H_2O_2, and their ^{35}S-content was determined by scintillation counting. For each protein analyzed, its radioactivity in the Ca^{++}-soluble fraction is expressed as a percentage of its radioactivity in the triton-soluble + Ca^{++}-soluble fractions. The data shown are means ± standard errors ($n = 4$).

to assay for MAP.

In identifying MAP of neurons, we used the relatively stringent criteria originally described by Solomon et al. (33). With these criteria, a protein will be identified as a MAP if it (*a*) remains associated with cytoskeletons prepared with triton-containing microtubule-stabilizing buffers, (*b*) is released from triton-insoluble cytoskeletons by incubation in microtubule-depolymerizing buffers, (*c*) is dependent on microtubules for its presence in Triton X-100-insoluble cytoskeletons, and (*d*) is able to co-cycle with brain microtubule proteins under in vitro conditions. We have identified 26 nontubulin proteins in cultured neurons that exhibit all of these properties (Fig. 1). We conclude that these proteins are associated with neuronal microtubules in situ. We have also identified several other proteins of relatively high molecular weight which satisfy criteria *a*, *b*, and *c* (Fig. 2) and it is likely that these proteins are also neuronal MAP.

The criteria outlined above would fail to identify as MAP proteins that have a Triton X-100-labile association with microtubules or proteins that associate with cytoplasmic components in addition to microtubules such that they are either Triton X-100-insoluble but cold-soluble in the presence or absence of microtubules, or Triton X-100 and cold-insoluble. In this regard we did not detect as putative MAP proteins comigrating with the HMW MAP-2 of brain (23, 32) in cold- or Ca^{++}-soluble fractions prepared from sympathetic neurons (Fig. 2). HMW MAP have been identified in neurons, using immunohistochemical procedures (9, 21). Our inability to detect MAP-2 in cultured sympathetic neurons may be because it has a Triton X-100-labile association with microtubules (10) or a sufficiently strong association with other cytoplasmic components, neurofilaments for example (1, 20), which renders it Triton- and cold-insoluble. It is also possible that the 240,000 molecular weight MAP (Fig. 2) is related to MAP-2, perhaps as a proteolytic fragment. Finally, sympathetic neurons may accumulate little, if any, MAP-2.

Analysis of the twenty-four MAPs in the molecular weight range of 60,000 to 76,000 by peptide mapping has led to the identification of four distinct sets of MAPs. Each set consists of several proteins that vary slightly in apparent molecular weight and isoelectric point, but that give rise to very similar or indistinguishable peptide maps (see Fig. 4). This latter observation suggests that the members of each set are closely related proteins. The possibility that posttranslational modification contributes to the variation within each set is supported by the observation that all except the most basic member of each set can be phosphorylated in situ (see Fig. 5). Also, in pulse-chase experiments (Fig. 6), the labeling patterns of MAP sets I, II, and IV changed in a manner suggesting that the more acidic members of these sets were derived from one or more precursors by posttranslational modification. The identity of the primary translation product(s) for each MAP set is unknown, although the pulse-chase and phosphorylation data are consistent with the possibility that it is the most basic member of each set. In considering the sequence of events by which the primary translation product(s) are modified to generate the other MAP, it may be relevant that the more acidic members of the MAP sets also appear to be their more heavily phosphorylated members (Fig. 5). Thus, it is possible that the more acidic members are derived by progressive phosphorylation of one or more of the more basic members. Experiments are in progress to identify the primary translation product(s) for each MAP set and define how they are modified to generate the other members of these sets.

Relationship between the MAP of Sympathetic Neurons and Brain

The high molecular weight class of brain MAP consists of two major proteins referred to as MAP-1 and MAP-2 (23, 32). As discussed above, we have not detected MAP-2 in cultured sympathetic neurons. However, we have identified a neuronal MAP that resembles MAP-1 in electrophoretic mobility on 1-D gels (Fig. 2), and we are currently attempting to obtain enough of this protein to compare it with brain MAP-1 by peptide mapping. Two other classes of MAP have been identified in brain, one designated LMW MAP and the other tau. The LMW MAP consist of two bands on SDS gels with apparent molecular weights of ~30,000 (1, 35). Two of the MAP identified in cultured rat sympathetic neurons have apparent molecular weights that closely resemble those of the LMW MAPs of brain and thus may correspond to these MAP. At present, however, we have no additional information regarding the possible relatedness of these proteins. The brain tau MAP consist of four closely spaced bands on SDS gels that range in apparent molecular weight from 55,000 to 70,000 (8, 14), and proteins antigenically related to brain tau have been detected in cultured neurons from fetal rat brain (9). The MAP in rat sympathetic neurons that have molecular weights of 60,000–76,000 show similarities and differences with respect to brain tau. The neuronal MAP resemble brain tau in that each consists of four distinct species that differ in apparent molecular weight. Also, in 2-D PAGE, each species of brain tau (8) and each set of neuronal MAP (see Fig. 1) consist of several proteins that vary in isoelectric point (also the tau-like MAP of neuroblastoma cells exists as sets of proteins with varying isoelectric points [24]), and, finally, the neuronal MAP behave like brain tau on phosphocellulose columns (M. M. Black, unpublished observation). Brain tau and the neuronal MAP differ in their heat stability and apparent molecular weights. These considerations suggest that while the four sets of neuronal MAP described here are tau-like in several respects, they are distinct from brain tau.

Distribution of MAP between Assembled and Unassembled States

When neurons were extracted under solution conditions that stabilized existing microtubules and also permitted quantitative separation of assembled and unassembled tubulin, some MAP partitioned predominantly with the fraction containing microtubules, while other MAP fractionated primarily with unassembled tubulin (see Fig. 7 and Table I). The

neuronal MAP that fractionate predominantly with microtubules also appear to be the most extensively phosphorylated MAP (compare Fig. 7 with Fig. 5), raising the possibility that phosphorylation may regulate the partitioning of the MAP between the fractions containing assembled or unassembled tubulin. Similar observations were originally reported by Pallas and Solomon (24) in their studies on MAP of neuroblastoma cells. The functional implications of these solubility properties of neuronal MAP are at present unclear. It is possible that they reflect the in vivo distribution of the MAP between assembled and unassembled states.

Localization of MAP in Neurons

Neurons contain complex populations of MAP. Several considerations indicate that the various MAP are highly localized to specific and distinct regions of the neuron. For example, HMW MAP have not been detected in axons by axonal transport studies (3, 34) or immunohistochemical procedures (21), but they have been observed in association with microtubules in dendrites (21). Although recent data would question whether these MAP are solely dendritic (35), it is clear that they are preferentially localized to dendrites. The following considerations suggest that the various tau species of MAP are also localized to different domains of the neuron. In the studies reported here, rat sympathetic neurons were shown to synthesize four distinct tau-like species. A similar set of tau-like proteins has also been observed in rat dorsal root ganglion neurons (Black and Kurdyla, unpublished observations). Recent studies on MAP in axons of guinea pig retinal ganglion cells have identified only two tau-like species (34). Although direct comparison of these observations is complicated by the differences in the systems examined, they suggest that only some of the total complement of tau synthesized by neurons are associated with axonal microtubules. Implicit in this interpretation is that the remaining species of tau are associated with cell body and/or dendritic microtubules. As mentioned in the introduction, axonal and dendritic microtubules also differ from each other in their pattern of organization. Under test tube conditions, MAP can cross-link microtubules with each other and with other cytoskeletal structures (13, 16, 20, 29). If MAP perform similar functions in vivo, then differences in axonal and dendritic MAP could contribute to the differences in the pattern of microtubule organization in these processes.

Axons and dendrites differ in their general morphological properties (25). Several lines of evidence indicate that the pattern of microtubule organization is a determinant of cell shape (26). On the basis of this evidence, it seems reasonable that the differences in microtubule organization in axons and dendrites may contribute to the differences in axonal and dendritic morphology. If this is correct, then selective partitioning of MAP between axons and dendrites may represent part of the mechanism that generates regional differences in the organization of the neuronal cytoskeleton and thereby neuronal morphology.

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