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Microtubule-associated tau protein function and expression during microtubule-dependent neuronal process extension

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Microtubule-associated tau protein function and expression during microtubule-dependent neuronal process extension

by

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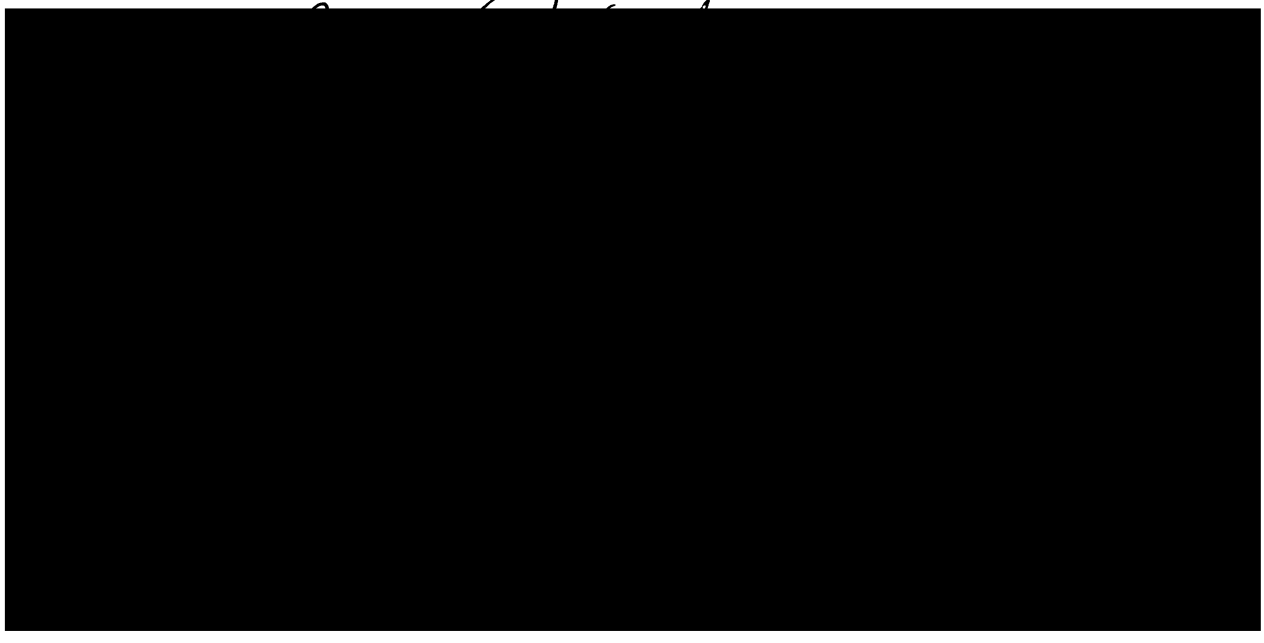

Microtubule-associated tau protein function and expression during microtuble-dependent neuronal process extension

by David Drubin

Abstract

Tau protein is a family of closely related polypeptides that binds to microtubules and promotes microtubule assembly *in vitro*. The relevance of *in vitro* studies on tau protein, however, to tau function *in vivo* rests on direct demonstration that tau protein binds to microtubules and promotes microtubule assembly in cells. Several different types of experiments aimed at increasing our understanding of tau protein's *in vivo* role are presented in this thesis.

Studies on tau function are complicated by the appearance of many tau isoforms which may be biologically significant or may be artifactually produced during isolation. Tau heterogeneity was found here to result from developmentally regulated expression of multiple tau isoforms in mouse brain. The most significant changes in tau expression occur during the stages of brain development involving extensive neurite sprouting, suggesting a role for tau in the reorganization of microtubules that occurs at this time. *In vitro* translation studies showed that the developmental change in tau expression can be largely accounted for by changes in tau mRNA. Despite evidence for post-translational modifications of tau *in vivo*, tau generated by *in vitro* translation shares with tau isolated from brain the ability to coassemble with tubulin into microtubules. Tau genomic organization was examined using tau cDNA probes generated from immuno-purified tau mRNA. Fewer tau genes were detected than the number of tau isoforms generated

by in vitro translation of brain mRNA. Tau heterogeneity may therefore be generated, in part, by RNA processing.

Tau was shown to be localized on microtubules in PC12 cells by immunofluorescence using anti-tau antibodies. In contrast, microtubules in fibroblast cells which contain no detectable tau do not stain with tau antibodies. However, after microinjecting purified tau protein into fibroblasts, tau partitions exclusively to microtubules. In addition, biochemical experiments showed that tau protein partitions with detergent-extracted cytoskeletons containing intact microtubules, but does not associate with cytoskeletons if the microtubules are specifically depolymerized with colchicine. Finally, treatment of PC12 cells with either colchicine, nocodazole, vinblastine or taxol, each of which acts specifically on microtubules, causes a post-translational modification (probably dephosphorylation) of tau. Thus, not only does tau interact with microtubules in living cells, but the state of tau is dependent on its interaction with microtubules.

The clonal PC12 cell line which extends neurites in response to nerve growth factor (NGF) was used to study microtubule assembly during neurite outgrowth. One effect of NGF was found to be the promotion of microtubule assembly during neurite outgrowth. Though NGF causes a general increase in total tubulin levels, the formation of neurites and assembly of polymer follow a slower time course. The increase in polymer and neurite extension closely parallel 10- and 20-fold inductions of tau and Microtubule-Associated-Protein-1 (MAP1). When NGF is removed, neurites disappear and both associated proteins return to undifferentiated levels. These data suggest that when tau and MAP1 are

induced they promote microtubule assembly and that they are therefore key factors regulating neurite outgrowth.

In memory of my mother and father

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"A man is judged by what he looks for, not by what he finds".

- Shamus

in "The Naive and Sentimental Lover"

by John le Carré

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CHAPTER ONE

INTRODUCTION

The Neuronal Cytoskeleton

The neuronal cytoskeleton consists of microtubules, actin filaments, and neurofilaments. Microtubules, arranged in densely packed parallel arrays in neuronal processes, are essential for neuronal process extension since drugs that specifically disrupt them cause growing neurites to retract (1,2,3). Actin filaments, found predominantly in the growth cone margin (4), are required for growth cone activity (5,6). The growth cone is responsible for substratum selectivity and for determining neurite shape. However, growth cone activity is not required for neurite extension, provided that an adhesive substrate is available (6). Thus, microtubules, but not actin filaments, are responsible for the outward force required to extend neurites. In fact, actin filaments provide an inward force opposing the force applied by microtubules in neurites (7), perhaps to give the growing neurite "plasticity". The function of neurofilaments, arranged in parallel arrays within nerve processes, is unclear, but they may act to strengthen the processes.

In addition to their role in neurite extension, microtubules are conduits for vesicular transport between nerve cell bodies and process tips (8,9). Importantly, dendrite and axon terminals must be biochemically distinct to receive or transmit signals, respectively. Establishment of this asymmetry requires selective targeting of components synthesized in the cell body to either dendrites or axons. This targeting could result from non-selective transport of vesicles on microtubules, with selective binding occuring at dendritic or axonal tips. Alternatively, vesicles could be transported selectively on either dendritic or axonal microtubules. The latter possibility is more

plausible since vast distances (up to one meter in man) separate cell bodies from process tips. Since dendritic and axonal microtubules are biochemically distinct (discussed further below), it is possible that vesicles contain receptors that allow specific interactions with one of the two microtubule types. In any case, understanding how a nerve cell acquires its elongated shape and biochemical asymmetry will require understanding how microtubule assembly is regulated spatially and temporally in differentiating neurons.

In Vitro Assembly of Neuronal Microtubule Proteins

Microtubules can be assembled in vitro from purified microtubule proteins (10). The tubulin "monomer", actually a heterodimer of 50,000 mol. wt. alpha and beta polypeptides, contains the information needed to form a microtubule since purified tubulin can assemble into microtubules (11). Microtubule assembly involves GTP hydrolysis (12) and is favored by physiological temperature (10). Low temperature and calcium ion favor disassembly (10).

When tubulin is purified from mammalian brain by successive cycles of temperature-induced assembly/disassembly and differential sedimentation, several accessory proteins copurify. Three of these proteins, tau (55-70,000 mol. wt.), MAP1 (350,000 mol. wt), and MAP2 (300,000 mol. wt), promote microtubule assembly in vitro (13-18). MAP2 and tau are present in approximately equimolar amounts in brain, representing about 1% (19) and 0.15% of total protein mass, respectively, with MAP1 being somewhat less abundant (Drubin, unpublished observation). The study of these proteins is complicated since each is characterized by considerable heterogeneity. Preparation of specific antisera and isolation of MAP cDNA clones is necessary to

elucidate the relationships between the polypeptides and to determine if the heterogeneity reflects the expression of functionally distinct isoforms, or if it is artifactually introduced during purification. The discussion below will focus on tau protein, the primary subject of my studies.

Physical and chemical properties of tau protein

When analyzed by SDS-PAGE, mammalian brain tau protein migrates as a series of 4 - 7 bands (20,21). The 4 bands originally observed have almost indistinguishable peptide maps and amino acid compositions (20,22), suggesting that they have closely related primary structures. Further evidence for the close relatedness of the different tau species is the observation that they share a common set of unique physical properties. Tau protein is stable to boiling (13,18), extremely elongated (but containing essentially no alpha-helical structure) (20), and is soluble in 2.5% perchloric acid (G. Lindwall, pers. comm.).

When tau protein is analyzed by two dimensional gel electrophoresis, the protein pattern begins to look hopelessly complex. Each band resolved on a one dimensional gel spreads to 5 - 6 discrete spots on two dimensional gels (20). Fortunately, this level of heterogeneity can be entirely accounted for by the existence of different tau phosphorylation states. When rat or bovine brain tau is extensively treated with alkaline phosphatase prior to analysis on two dimensional gels, the pattern is reduced to approximately one discrete spot for each of the tau species resolved on one dimensional gels (G. Lee, pers. comm.). In addition, the seven tau bands seen on one dimensional gels when tau is purified directly from brain without prior warm and cold cycling can be reduced to distinct sets of 4 bands of

faster mobility after phosphatase treatment, or slower mobility after kinase treatment (21). This result suggests that phosphorylation causes a conformational change in tau protein. Since extensive phosphorylation state heterogeneity is observed when tau is isolated without coassembly with tubulin, multiple phosphorylation states of tau may exist in brain. Caution must be applied when interpreting these results, since considerable phosphorylation state heterogeneity can be introduced during sample preparation (G. Lee, pers. comm.).

In vitro studies indicate that tau is a substrate for a calcium and calmodulin-dependent protein kinase in brain extracts (23), and for both cAMP-dependent and -independent protein kinases in microtubule protein preparations (D. Gard, pers. comm.).

Effect of tau protein on microtubule assembly

Tau protein acts both catalytically, promoting microtubule nucleation and elongation, and stoichiometrically, increasing the extent of microtubule assembly (14,24). Tau saturation occurs at about one tau per ten tubulin molecules (24). Because of the highly elongated shape of tau protein and its ability to interact with tubulin ribbons (interpreted to represent microtubule protofilaments) (25), it has been proposed that tau protein acts to increase microtubule nucleation, elongation, and stability by simultaneously binding several tubulin subunits along microtubule protofilaments (thirteen protofilaments comprise a microtubule). Because tau has been proposed to lie along a protofilament, it may contain a repeated tubulin binding domain. Such a feature may be observed when the tau amino acid sequence is determined.

Recent experiments using tau protein, microtubule organizing centers (centrosomes), and MAP-depleted tubulin have shown that, in

addition to increasing the rate and extent of nucleated microtubule assembly, tau increases the rate at which tubulin monomers associate with polymer (M. Kirschner, pers. comm.). It also increases dramatically the number of microtubules nucleated from a centrosome (T. Mitchison, K. Matlack, pers. comm.). Further experiments are needed to determine the effect of tau protein on the recently described "dynamic instability" (26) behavior of microtubules. In the presence of tau, GDP ends should have an increased probability of re-capping with GTP-tubulin. Therefore, it is likely that the extent of "catastrophic" disassembly events would be reduced.

Since microtubules in neuronal cells are coated with tau protein (see Chapter 3), it is reasonable to assume that tau imparts some of the same properties to microtubules in vivo as it does in vitro. Thus, microtubule stability should increase with increasing amounts of tau protein. Also, the rate of microtubule nucleation from organizing centers, and steady state levels of assembled tubulin, should be proportional to tau concentration.

In cells, tau binding should also make microtubules responsive to various cellular regulatory signals. As mentioned above, tau is a substrate for cAMP-dependent and cAMP-independent protein kinases in microtubule protein, and for the brain calcium and calmodulin-dependent protein kinase in vitro. One possible consequence of tau phosphorylation is suggested by the observation that phosphorylated tau is less active than unphosphorylated tau in promoting microtubule assembly (21). Thus, activation of kinases by calcium ion, cAMP, or other signals, could result in altered affinity of tau for microtubules. In addition, Ca^{2}+-calmodulin binds tau in the presence of micromolar Ca^{2}+ (27). As a

result, tau-induced microtubule assembly is sensitive to physiological calcium ion levels, while MAP2-induced microtubule assembly is not (28). Since tau is predominantly axonal (29) and MAP2 is predominantly dendritic (30,31), microtubules in the two compartments can respond differently to these signals. Furthermore, the different tau isoforms may respond differently to various physiological signals. If the tau isoforms are segregated to different microtubules, microtubules within an axon could respond to signals differently.

An alternative function for tau protein is to serve as an attachment site for other molecules localized to microtubules. One possibility is that soluble proteins associate with microtubules through tau protein, just as the cAMP-dependent kinase regulatory subunit is localized to microtubules through its interaction with MAP2 (32). Another possibility is that tau cross-links microtubules with actin filaments or intermediate filaments. It is not likely that tau directly cross-links two filament systems since it has a low profile on microtubules as evidenced by electron microscopy. This is in sharp contrast to MAP1 and MAP2 which interact with microtubules through a small domain, perhaps solely for anchorage, leaving most of the molecule to project outward from microtubules (33,34). Indeed, while both tau and MAP2 bind microtubules and cross-link actin filaments, only MAP2 cross-links microtubules with actin filaments (35). It still remains possible, however, that tau can cross-link microtubules with other filaments through intermediary molecules. One intriguing possibility is that microtubule-bound tau protein could interact with actin filament-associated troponin C, known to be homologous to calmodulin,

through its calmodulin-binding domain. Such a crosslink would be calcium-regulated.

In conclusion, tau protein bound to microtubules in vivo should influence the properties of the tubulin lattice. Also, because tau protein has been interacting with microtubules over a long stretch of evolutionary time (22, also see Chapter 4), it is likely that proteins have evolved to use tau as a binding site on microtubules.

Approaches to studying tau function in vivo

The in vivo role of tau, or any other MAP, has not been demonstrated. Ideally a pharmacological agent that binds to tau, or a tau mutation, could be used to study tau function in vivo. These, however, do not exist. Alternatively, state-of-the-art techniques such as microinjection of tau, tau antibodies or tau antisense mRNA might be used in an appropriate system to interfere with tau function. First, however, an easily manipulated system must be developed and characterized.

A promising system for the study of tau function is neuron differentiation. During brain development, tubulin levels reportedly remain constant (36) even though microtubule-dependent neurite extension involves microtubule assembly (see Chapter 4). Thus, it appears that a reserve of monomeric tubulin subunits is assembled during neurite extension. The expression of MAPs with SDS-gel mobilities similar to tau changes in brain during neurite sprouting (37). The newly expressed tau species could promote neurite microtubule assembly. Consistent with the conclusion that tau promotes microtubule assembly during neurite extension, the ability of tau to promote microtubule assembly increases during brain development (38). However, due to the heterogeneous nature

of brain tissue, it is not known whether different tau species are expressed in different cell types at different times, or if different taus are expressed developmentally within single cells. More importantly, it is not known if, within a given cell, tau expression correlates precisely with neurite extension.

Several clonal cell lines synchronously extend microtubule-filled neurites in culture, thereby providing a homogeneous system for biochemical studies. In one neuroblastoma cell line, differentiated cells have higher levels of assembled tubulin, but the same levels of total tubulin, as undifferentiated cells (39). Such a system is well-suited for studying the role of neuronal MAPs in regulating microtubule assembly during neurite extension. Consistent with the hypothesis that MAPs promote microtubule assembly during neurite extension, MAP activity is elevated in differentiated neuroblastoma cells (40), and MAP1 levels are elevated in differentiated N115 neuroblastoma and PC12 pheochromocytoma cells (41,42). By careful quantitation, tau, MAP1 and MAP2 levels can be compared with levels of assembled tubulin in these systems. Finally, the relevance of any experiments designed to test the effect of tau protein on microtubule assembly in a cell line rests first on the demonstration that tau associates with microtubules in that cell line. Once a system is described, antibodies and antisense mRNAs can be utilized to interfere with MAP function.

This thesis consists of three chapters reporting experimental studies aimed at increasing our understanding of tau protein's <u>in vivo</u> role. In Chapter 2 the preparation of specific anti-tau antibodies and tau cDNA clones is described and the molecular basis of tau protein

heterogeneity in mouse brain is explored. In Chapter 3 the association of tau protein with microtubules in living cells is demonstrated using biochemical and immunocytochemical techniques. Finally, the results presented in Chapter 4 show that the induction of neurite outgrowth in PC12 cells by nerve growth factor involves increases in microtubule assembly and the induction of tau protein, suggesting that tau protein promotes microtubule assembly *in vivo*.

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CHAPTER TWO

Studies on the expression of the microtubule associated protein, tau, during mouse brain development with newly isolated cDNA probes

ABSTRACT

Tau protein is a collection of closely related polypeptides that associate with microtubules *in vivo* and stimulate their assembly *in vitro*. Using an affinity purified antiserum against bovine brain tau protein, we found that the number and amount of tau polypeptides changes dramatically during mouse brain development. The different forms appear to result from changes in tau mRNA since *in vitro* translation products reflect the qualitative and quantitative changes found *in vivo*. In order to study the mRNA and genomic complexity of tau protein we isolated embryonic mouse tau cDNA clones. With these probes we have determined that embryonic tau protein is translated from a 6 kb mRNA that persists throughout brain development.

Microtubule assembly *in vitro* is promoted by a set of proteins called microtubule associated proteins (MAP). These proteins coassemble with tubulin into microtubules, and have been located on microtubules *in vivo* (8,9). Two major classes of microtubule associated proteins have been distinguished, the lower molecular weight tau proteins of 55-62 kd and the higher molecular weight MAP proteins of approximately 300 kd (5, 19, 23, 30, 32). In addition, MAP proteins of intermediate molecular weight have been described in primate cells (2). Within each class of proteins there is considerable heterogeneity.

Tau protein was originally found to be quite heterogenous, appearing as 4 or 5 major bands in mature pig brain when examined on one dimensional SDS-polyacrylamide gels and 5 times that number on 2D gels (5). Despite this heterogeneity, the pig brain tau species which are resolved on one dimensional gels are virtually indistinguishable when compared by peptide mapping and amino acid analysis (5,6). Recently a 50 kd coated vesicle protein (26) as well as neuroblastoma microtubule-associated proteins of 69 and 80 kd (10), have been shown to be related to brain tau proteins.

The close relatedness of the different tau polypeptides suggests they share a function, such as binding to microtubules. The existence of many different forms of tau protein on the other hand has raised questions as to the biological significance of the heterogeneity. Does this heterogeneity represent functional differences in the proteins, strategies for cellular localization, developmental regulation, or even steps in degradation? These are important questions, but the extreme heterogeneity of tau protein itself complicates considerably the study of its structure and activity.

To begin to examine the function of tau protein and to find a role for its heterogeneity, we have studied changes in tau protein during mouse brain development and the level at which this heterogeneity is generated. As a further step in understanding the structural basis of this heterogeneity, we have isolated cDNA probes for an embryonic form of tau protein in mouse brain. Using these probes we have begun to study the relationship among the various forms of tau protein and how tau protein expression is regulated.

MATERIALS AND METHODS

Analysis of Brain Tau Proteins

Brain extracts were prepared immediately after sacrificing ICR mice or several hours post-mortem for beef brain, by homogenizing tissue in ice cold PB buffer (0.1M morpholinoethanesulfonic acid, 0.5 mM $MgCl_2$, 2mM EGTA, 0.1 mM EDTA, 1mM β-mercaptoethanol, pH 6.4) supplemented with 0.5 mM PMSF, 1 mM aminoacetonitrile and 10 mM benzamidine HCl, in a motor -driven teflon pestle. The homogenate was centrifuged for 30 min., 4° C at 40,000 rpm in a Beckman 50Ti rotor. An aliquot was assayed for protein (21), and the remainder was boiled in SDS sample buffer and stored at -20° C. Microtubule protein (MTP) was purified by the Weingarten et al modification (32) of the Shelanski et al. procedure (29) and PC-tubulin by chromatography on phosphocellulose (32). Tau protein was purified by the method of Herzog and Weber (17).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli (20) and immunoblotting experiments were carried out by the procedure of Burnette (3). Preparation of the affinity-purified bovine tau antiserum was described previously (26). Immune complexes were detected with 10^6 cpm per ml of iodinated (18) IgG fraction of goat anti-rabbit Ig antiserum (Cappel Laboratories, Cochransville, PA). Kodak X-O-Mat-AR film was used with Cronex Lightning Plus intensifying screens for autoradiography.

Translation of Brain mRNA and Characterization of Resulting Products

To purify RNA, 5 g of brain was homogenized in 40 ml of homogenization buffer (3M LiCl, 6M Urea, 20mM Tris-Cl [pH 7.4], 10mM $MgCl_2$, 1% β-mercaptoethanol) in a Polytron homogenizer. After gentle shaking for 15 min. at room temperature, particulate material was

pelleted for 5 min. at 10,000 rpm in a Sorvall centrifuge. The homogenate was incubated overnight at 4°C and precipitated RNA was collected by centrifugation at 4,000 rpm for 15 min. The pellet was washed twice in homogenization buffer, resuspended in 1 ml of 10mM Tris -Cl [pH 7.4], 10mM NaCl, then extracted once with an equal volume of phenol.

Poly-A^+ RNA was isolated from total RNA by one cycle of chromatography on oligo (dT)-cellulose. PolyA+ RNA recovery was assayed by the ability of the poly A to hybridize to ^{3}H-polyU and thus protect the ^{3}H-polyU from a single strand-specific nuclease. An approximate 100 fold molar excess of ^{3}H-polyU over poly A was incubated with polyA+ RNA in 2XSSC (1XSSC = 150mM NaCl, 15mM Nacitrate) at 45°C for 15 min., then on ice for 5 min. RNase A was added to 10µg/ml for 15 min. on ice. 100ng of carrier DNA was then added, TCA was added to 5%, and the precipitate containing protected ^{3}H-polyU was collected on a Whatman GF/C filter. The filter was dried and scintillation-counted in Aquasol (NEN).

To fractionate mRNA by molecular weight, 10 µg in 40 mM methylmercuric hydroxide was run at 50 V for 5 h. on 1% low-melting agarose (Sea Plaque) gels containing 5 mM methylmercuric hydroxide. 1 mm gel slices were placed in an equal volume of 1% β-mercaptoethanol, 0.2 % SDS, 10 mM Tris-Cl [pH 7.4], 1 mM EDTA, 0.1 M NaCl, and boiled for 20 s. Samples were immediately vortexed for 1 s., phenol extracted until interface material disappeared, and ethanol precipitated twice.

mRNA was translated in a rabbit reticulocyte lysate system (BRL) containing [^{35}S]-methionine (25). Translation products were either directly analyzed by SDS-PAGE followed by fluorography with EN^{3}HANCE (NEN) or were first immunoprecipitated for tau. To immunoprecipitate

tau proteins, 30 µl of translation reaction was boiled with 4.5 µl 10% SDS and added to 105 µl of LB (25 mM Tris-Cl [pH 7.4], 0.4M NaCl, 0.1 % deoxycholate, 1% NP40). This mixture was used to resuspend 30 µl of washed and pelleted S. aureus cells (Pansorbin, Calbiochem). After 10 min. at room temperature, the samples were centrifuged 2 min in an Eppendorf centrifuge. The precleared supernatants (140 µl) were transferred to tubes containing 55 µl PBS and 125 µl affinity-purified tau antiserum or an equivalent amount of nonimmune rabbit IgG (Cappel Laboratories). After 20 min. at room temperature, 30 µl of a 10% suspension of S. aureus cells, washed once in PBS and resuspended in PBS supplemented with 10 mg per ml BSA and 1 mg per ml methionine, was added. After 5 min. at room temperature, the cells were collected by centrifugation. The cells were washed twice in 200 µl LB with 0.5 % SDS and once in 200 µl 10 mM Tris-Cl [pH 7.4], 5 mM EDTA, each time collecting cells by centrifugation through a 0.6 ml cushion of 0.25 M NaCl, 25 mM Tris-Cl [pH 7.4], 1% Triton X-100, 0.5% sodium deoxycholate, 5 mM EDTA and 1 M sucrose. The pellets were resuspended in 40 µl of SDS sample buffer. After boiling, samples were centrifuged and 5 µl of supernatant was counted in Aquasol (NEN) while the rest was analyzed by SDS-PAGE.

Coassembly of translation products into microtubules was done by incubating 60 µl of translation reaction with 400 µl PC-tubulin in PB buffer in the presence of 1 mM GTP and 25 mM taxol for 30 minutes at 30° C. 100 µl of microtubules were pelleted through 0.5 ml 50% sucrose in PB buffer for 60 min. at 48K rpm in a Beckman SW50.1 rotor carrying microtube inserts. The microtubes were cut within the sucrose cushion and the sucrose solution was aspirated. The pellet was then resuspended

in 300 µl PB supplemented with 1 M NaCl, 2mM DTT and 75 µl 20 mg/ml MTP. The sample was boiled for 5 min., cooled on ice for 5 min., and centrifuged for 20 min. in an Eppendorf centrifuge. Soluble proteins were TCA precipitated and analyzed on SDS-PAGE.

Isolation and Characterization of Tau cDNA Clones

Polysomes were prepared by homogenizing fresh mouse brains (frozen on dry ice until all brains were collected) in 3.5 ml PHB (50 mM Tris-Cl[pH 7.4], 25 mM NaCl, 5 mM $MgCl_2$, 25 mM sucrose, 0.5% deoxycholate, 1% NP40, 0.2 mg per ml heparin, 5 µg/ml emetine-HCl, 5 µg/ml cycloheximide, 0.5 mg/ml yeast RNA) per gram of brain at 4°C using a teflon homogenizer. The homogenates were spun at 15,000 rpm for 10 minutes in oven-baked Corex tubes at 4° C then immediately loaded on a discontinuous sucrose gradient in PPB (25 mM Tris-Cl [pH 7.4], 0.15 M NaCl, 5 mM $MgCl_2$, 0.1% NP40) with 0.5 ml, 3 ml and 1 ml steps of 0.5 M, 1.17 M, and 2.5 M sucrose, respectively, in a Beckman SW41 rotor for 2 h at 4° C. The gradient was aspirated to the top of the 2.5 M sucrose plug where the polysomes banded. Polysomes were collected and frozen in liquid nitrogen until used. We obtained 50 Absorbance units (at 260 nm) of polysomes from 4 g brain.

Polysomes containing tau mRNA were purified by the method of Shapiro et al. (28). RNase-free tau antiserum was made by affinity purifying tau antibodies (26) in RNase-free media. 50 Absorbance units of polysomes yielded about 80 ng of tau mRNA.

Using the protocol described by Goddard et al. (15), immunoselected tau mRNA was used to create a cDNA library in DH1 bacteria. We obtained about of 2.5 ng of double-stranded size-selected cDNA from 40 ng of mRNA.

Colonies were stored and screened as described by Gergen et al. (13). Radiolabeled probes from tau or total mRNA were made by utilizing reverse transcriptase in first strand synthesis with [α-^{32}P] dCTP. Hybridization and washing conditions were as described below for DNA and RNA blots.

Hybrid-selection experiments were carried out with 1 mm^2 filters cut from 1 cm^2 filters prepared with 20 µg of plasmid DNA as described by Cleveland et al (7). Filters were preincubated for 4 h at 47° C in 250 µl HM (55% formamide, 10 mM PIPES [pH 6.4], 0.5 M NaCl, 2 mM EDTA) containing 5 mg/ml yeast tRNA, then in 125 µl of an identical solution containing 15 µg mouse poly-A^+ RNA (preheated for 1 minute at 65° C) for 4 h at 47° C. The filters were washed 3X with 0.2X SSC (1X SSC = 150 mM NaCl, 15 mM Nacitrate) 0.1% SDS, 1 mM EDTA, and 5X with 0.2X SSC, 1 mM EDTA, both at 47° C. Bound RNA was eluted by boiling filters in 200 µl 1 mM EDTA, 70 µg/ml tRNA for 90 sec. and quick freezing in dry ice/ethanol. After thawing the filter was removed and 20 µl 2M potassium acetate [pH 6.4] was added and the solution was centrifuged to remove any residual SDS precipitated by the potassium ion. After ethanol precipitation the RNA sample was added to an in vitro translation system.

Electrophoresis and Blotting of DNA and RNA

RNA was fractionated on 0.9% agarose gels containing formaldehyde, as described by Goldberg (16), and transferred directly to nitrocellulose in 20X SSC. DNA was fractionated on 0.7% agarose gels and transferred to nitrocellulose as described by Southern (31). RNA and DNA blots were hybridized at 42° C in 5X SSC, 50% formamide, 150 µg/ml calf thymus ssDNA, 0.1% SDS, 0.2% BSA, 0.2% ficoll, 0.2%

polyvinylpyrrolidone. Probe generated by nick translation (27) was hybridized for 24-48 hours. Blots were washed 2 hours at 50° C in 0.1X SSC, 0.1% SDS.

RESULTS

Analysis of Cow and Mouse Brain Tau Proteins

Affinity-purified antiserum against bovine brain tau protein was used on nitrocellulose transfers to analyze the forms of tau protein present in cow and mouse brain extracts. Fig. 1 (a-c) shows tau polypeptides from cow brain. In lane a the soluble brain extract was probed with antibody to tau. Six major bands with molecular weights between 47 and 61 kd were observed. The tau antigens in microtubule protein and in purified tau protein are shown in lanes 1b and 1c . The lowest band in the total brain extract, lane a, disappears as tau protein is purified by coassembly into microtubules (lane b). An additional major low molecular weight antigen is lost in the further purification of tau (lane c). Low molecular weight tau polypeptides were also detected when tau protein was purified directly from brain without coassembly with tubulin (4).

Major changes in tau antigens occur during mouse brain development. Between 12 and 15 days of fetal development (Fig. 1d and e), tau polypeptides of 47-50 kd (a doublet in lighter exposures) increase in abundance relative to total protein, while between 5 and 15 days after birth (Fig. 1f and g) these polypeptides decrease and polypeptides ranging up to 62 kd arise. Finally, in adult brain (Fig. 1h) the overall levels of tau antigens decrease. The tau antigens are highly enriched in cerebrum (Fig. 1i) over cerebellum (Fig. 1j).

The antigenic relationship among these proteins is supported by two experiments (data not shown). First, an independently isolated tau antiserum raised against hog tau protein (8) gave identical staining of proteins to that seen in Fig. 1d-h. Second, the gel-purified uppermost

cow tau protein (Fig. 1c) competed away the binding of our anti-serum to all the tau antigens present in adult mouse brain.

Characterization of the heterogeneity of tau by in vitro translation of tau mRNA and the purification of tau mRNA

To study the heterogeneity of tau protein at the mRNA level, tau protein was immunoprecipitated from in vitro translation reactions using RNA from various sources (Fig. 2). The four tau polypeptides resulting from the in vitro translation of cow brain mRNA (Fig. 2, lane 1) resemble those seen in highly purified cow tau protein (Fig. 1, lane c). The patterns of tau polypeptides synthesized with 6 day mouse mRNA (Fig. 2, lane 5) and adult mouse mRNA (Fig. 2, lane 3) again resemble those seen in total brain extracts (Fig. 1, lane f and h). They are, however, not identical suggesting that the protein products may be modified or have differential stability in vivo. Both the translation of 6 day mouse brain mRNA and the immunoblot of proteins from the same developmental stage show 2 major species at a molecular weight of 47 to 50 kd. In the adult brain the translation reaction shows decreased amounts of a tau species at 47-50 kd. Translation of 6 day and adult mRNA both show proteins of 62 kd although no 62 kd protein was detected in early mouse extracts (Fig. 1, lane f). Adult mRNA directs the synthesis of additional faint translation products of 50 - 62 kd which may correspond to peptides in adult brain of that size.

To study the complexity of tau on the RNA level it was necessary to isolate a tau cDNA clone. We chose to use 6 day old mouse brain as a source of mRNA, since the immuno-blotting experiments (Fig. 1) and the translation experiments (Fig. 2) showed that tau protein and tau mRNA levels are highest at that stage. First, two characteristics of tau

protein, its ability to efficiently coassemble into microtubules (4) and its stability to heat treatment (32) were used to further demonstrate that the immunoreactive translation products from 6 day mouse brain mRNA were tau. Before immunoprecipitation the translation products are seen as a heterogenous array of proteins, but 2 - 4 closely spaced bands are seen after immunoprecipitation with antitau serum (Fig. 2, lane 7). When the entire translation mixture was added to tubulin, which was polymerized into microtubules, the major translation products which pelleted were α and β tubulin (Fig. 2, lane 8). When the microtubule pellet was boiled, the supernatant (Fig. 2, lane 9) was highly enriched in tau proteins (compare lanes 7 and 9). This experiment reinforces the conclusion that the major translation products recognized by tau antiserum have the properties of tau proteins.

In order to enrich for tau mRNA to make cDNA clones, polysomes isolated from 6 day mouse brain were reacted with tau antiserum and the polysomes with nascent tau peptides were isolated by passage over a protein A-Sepharose column.

The extent of purification of tau mRNA can be estimated by quantifying the proportion of protein immunoprecipitated from translation products directed by total mouse brain mRNA or by immunopurified tau mRNA. Separate experiments showed that the immunoprecipitation is 30% efficient. Of the 864,000 cpm incorporated using total brain mRNA, the tau antibody precipitates only 390 cpm, shown in Fig. 3, lane 2, indicating that tau mRNA represents approximately 0.15% of the total translatable mRNA. Preimmune serum precipitates nothing (lane 1). In the absence of added mRNA the reticulocyte lysates incorporate no counts which can be

immunoprecipitated by tau antiserum (lane 4). However, out of only 29,000 cpm derived from translation of immunopurified tau mRNA, 900 cpm (representing 10.2% of total translatable mRNA after correction) can be immunoprecipitated by tau antiserum (lane 6) indicating a 60 fold purification. We therefore expected 10% of the clones derived from this mRNA to contain tau sequences.

Isolation and characterization of tau cDNA clones

Bacterial clones containing tau cDNA were identified as those which hybridized to radiolabeled probes made from immuno-purified tau mRNA but not from total 6 day mouse brain mRNA. In close agreement with the percent of positive clones we expected (see above), 11% of the colonies (81 out of 737) were positive. These colonies contained cDNA of two types which did not cross-hybridize when examined by blot analysis (data not shown). A large representative of each type (pTA1 and pTA2), with inserts of 1300 and 1900 bp was used in each of the experiments described below.

To confirm that plasmids pTA1 and pTA2 contain sequences homologous to tau mRNA, they were fixed to nitrocellulose filters and used to select tau mRNA out of total 6 day mouse brain mRNA. The amount of tau mRNA bound to each filter was assayed by translating the mRNA and immunoprecipitating the resulting translation products with tau antiserum. We included control filters in the experiment which did not contain putative tau clones. Since under our incubation conditions each filter non-specifically binds some of the total brain mRNA, we expected to see a faint tau protein immunoprecipitate from the control filters. However, we expected that if a filter contained a tau cDNA clone, the immunoprecipitated tau signal should be increased over background. Fig.

4, lane b shows the immunoprecipitated signal from 250,000 cpm of the translation products directed by total brain mRNA, while lanes c-f show signals from only 60,000 cpm of translation products directed by RNA selected by cloned DNA. Lane c shows the background signal from mRNA selected by a control filter containing a β tubulin cDNA clone, while lane f shows another control with a blank nitrocellulose filter. The signal in lane b is about four times as intense as that in lanes e and f, which is expected since four times as many cpm were used in lane b as were used in lanes c and f. When plasmids pTA1 and pTA2 were tested, each gave strong tau signals when compared to controls (Fig. 4, compare lanes d and e to lanes c and f). Note that the signals in lanes d and e are more intense than the signal in lane b, despite the fact that four times as many cpm were used in lane b as were used in lane d and e. This result is highly reproducible.

Complexity of Tau mRNA and Genes

One possible explanation for the failure of pTA1 and pTA2 to cross hybridize is that they represent different parts of a large mRNA. To investigate this possibility, we probed mRNA and genomic DNA blots with pTA1 and pTA2. Fig. 5 shows pTA2 hybridizing to 5, 10, 15, 20 day and adult mouse brain mRNA. A single band of 6 kb appears in every sample and there is little change in relative abundance. The hybridization pattern is identical when pTA1 is used instead of pTA2 (not shown, but for related data see Fig. 7).

To confirm that the tau translational activity resides in a 6 kb mRNA, 6 day mouse brain mRNA was fractionated on a denaturing agarose gel, eluted from slices, translated, and the resulting products were immunoprecipitated. Fig. 6 shows that all of the tau translational

activity resides in the 5.3 kb to 7 kb range with a peak at 6 kb. No appreciable tau translational activity is found at lower molecular weight. To answer the question of whether the two tau clones represent different genes whose mRNA products cannot be resolved by gel electrophoresis or represent different regions of the same gene, mouse genomic DNA digested with different restriction enzymes was separated by electrophoresis, blotted and probed with either pTA1 (Fig. 7A, lane 1-3), or pTA2 (Fig. 7A, lane 4-6). Both probes hybridize to the same major band in Bam HI, Hind III and EcoRI digests. However, pTA2 hybridizes faintly to several additional bands not hybridized by pTA1.

As a further test of the relationship between pTA1 and pTA2, we tested for related genomic sequences in species other than mouse, as suggested by the conserved nature of tau protein (6). As shown in Fig. 7B, pTA1 and pTA2 hybridize to the same fragments in mouse, human and chicken genomic DNA. However, pTA2 hybridizes to human DNA more strongly than pTA1, and, in addition, hybridizes to frog, and perhaps Drosphilia DNA, suggesting it may contain more highly conserved sequences than pTA1.

DISCUSSION

Tau protein from hog brain was originally described as four or five closely related polypeptides of 55 - 62 kd which facilitate the *in vitro* assembly of tubulin into microtubules. Since the purification of tau protein involved successive warm and cold incubations of crude brain extract, it was possible that tau heterogeneity arose adventitiously *in vitro*. We have examined tau polypeptides directly in brain extracts made in the presence of protease inhibitors and boiled in SDS (Fig. 1). Not only does tau heterogeneity exist in crude cow and mouse brain extracts, but the heterogeneity is developmentally programmed in mouse. Developmentally regulated changes in tau protein may be a general feature of mammalian brain development since similar changes were observed in purified rat and guinea-pig polypeptides (12, 21). We do not know the significance of tau heterogeneity, but one tau polypeptide in crude cow brain (Fig. 1, lane a) does not coassemble into microtubules, suggesting that it may not interact with microtubules in cells. Also, a 50 kd cow brain coated vesicle protein is immunologically related to tau protein (26).

How does tau protein heterogeneity arise? One possibility is that all tau polypeptides are cleaved from a common precursor protein. This is not likely since much of the cow and mouse tau protein heterogeneity observed in brain extracts (Fig. 1) can be generated by *in vitro* translation of corresponding mRNAs (Fig. 2). This is particularly clear for the translation of cow brain mRNA which, as shown in Fig. 2, lane 1, gives 4 major species corresponding to the upper 4 bands in Fig. 1, lanes a - c. In mouse, changes in the 47 - 50 kd tau polypeptides from 5

day old to adult mouse (Fig. 1, lane f and h) are reflected by translating the corresponding mRNAs (Fig. 2, lanes 5 and 3). However, the 62 kd tau polypeptide present in adult, but not in 5 day mouse, appears to be synthesized by both mRNAs. This may reflect a higher turnover of the 62 kd mouse tau early in brain development, or the masking of its mRNA until later in development. The lowest two cow tau species seen in Fig. 1, lanes a and b, however, are either not primary translation products, or are not antigenic when translated. It has been reported that all rat tau species can also be generated by in vitro translation and that some rat tau mRNAs appear in the brain before their corresponding proteins accumulate (14). These results suggest that different mRNAs exist for different tau species.

We have studied embryonic mouse tau protein. Fig.1, lanes d - j, shows that embryonic tau, which occurs as a doublet of 47 - 50 kd, peaks in abundance around the time of birth and diminishes through development, being largely replaced by higher molecular weight tau species. When embryonic tau is synthesized in in vitro translation reactions, two to four proteins of 47 - 50 kd are observed (Fig. 2, lane 7). Characteristic of tau proteins, these proteins efficiently coassemble with tubulin into microtubules and are thermal-stable. It is interesting that the primary translation products should efficiently coassemble into microtubules since neuroblastoma and pheochromocytoma tau proteins are preferentially phosphorylated when associated with microtubules (24). If phosphorylation is necessary for coassembly, the primary translation products must be phosphorylated in the reticulocyte lysate or by the added tubulin prior to assembly. Further analysis is necessary to see if this occurs.

In order to study the nature of tau heterogeneity on a genetic level we isolated cDNA clones for embryonic mouse brain tau protein. Two criteria were used to first identify these clones. First, they were positive when screened with a probe made from immuno-selected tau mRNA, but not when screened with probe made from unselected total brain mRNA . Second, the clones hybridize to tau mRNA as judged by hybrid-selected translation experiments. That two non- overlapping sets of clones were isolated may be the result of an internal priming site for reverse transcriptase in the cDNA reaction. Perhaps the mRNA has two stable configurations which expose different priming sites. Alternatively, the S1 nuclease digestion after second cDNA strand synthesis (15) may have cut the tau cDNAs in half if an AT rich region was present in the middle.

The embryonic tau cDNA clones were used to study the source of tau heterogeneity on the genetic level. We were surprised to find that embryonic tau protein is made from a 6 kb mRNA since only 1.3 kb of coding sequence is required for a 50 kd protein. Even more surprising is the fact that only a single mRNA was detected throughout brain development despite the fact that two tau species of 47 - 50 kd occur in early mouse brain and more species arise later. How then, can we account for the heterogeneity of tau protein? One possibility is that our probe recognizes multiple mRNAs which are unresolved on our gels. The mRNAs could be the products of different genes or could result from developmentally regulated differential mRNA processing of a common precursor mRNA. Differential mRNA splicing accounts for different forms of several proteins (1, 11). Another possibility is that different tau mRNAs exist for different tau polypeptides which are resolved on our

gels, but that our probes are specific for only one of those mRNAs. We have cloned 3.2 kb of a 6 kb mRNA. If our clones do not contain coding sequence, only the single homologous mRNA would be recognized. We think that our probes do contain coding sequences since they are phylogenetically conserved. Noncoding sequences tend not to be conserved (7), although there are execeptions. If multiple mRNAs do exist through development, we may be able to detect them by doing RNA blots at lower hybridization stringencies.

When we cut the 6 kb 6 day mRNA out of a gel and translated it (Fig. 6) we observed the same degree of heterogeneity seen in early mouse brain (Fig. 1, lane f) where a 47 - 50 kd tau doublet occurs. (What appears to be a single species above 47 kd in Fig. 6 is actually a doublet on close inspection.) This doublet may be generated by two mRNAs which comigrate at 6 kb. Alternatively, the doublet could be made from a single mRNA. How could two polypeptides arise from a single mRNA? If our cell-free translation system is faithfully translating tau protein, the heterogeneity may be due to correct in vivo post-translational modifications (which also occur in the reticulocyte lysate). Alternatively, there may be built-in imprecision in the translational initiation and/or termination signals on the tau mRNA. Finally, although we know of no precedent for a non-viral polycistronic mRNA in a eucaryote, we note that the tau mRNA is large enough to code multiple polypeptides of 50 kd.

In the case of cow tau protein, we have preliminary evidence suggesting that there are multiple 6 kb tau messages (Drubin and Kirschner, in preparation). On Northern blots of cow mRNA probed with mouse tau cDNA probes, we see a single band comigrating with the mouse

tau mRNA at 6kb. When we fractionated cow polyA+ RNA and translated it as was done for mouse mRNA in Fig. 6, all of the four tau bands seen in Fig. 2 (lane 1) were translated from two of the fractions, containing mRNA of about 7 - 5 kb. However, the translation products resulting from the fraction containing larger mRNA were enriched in the uppermost tau band. This result suggests that separate mRNAs exist for different cow tau species. In addition, this result demonstrates that our mouse tau cDNA probe is able to hybridize to cow tau mRNA. In summary, we have shown that tau hetrogeneity is developmentally programmed and is not principally due to proteolysis during purification; the possibilities for generating this heterogeneity are extensive but the tau cDNA clones and high titer antisera against the protein will allow us to answer these questions.

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FIGURE LEGENDS

Figure 1

Immunoblot analysis of cow (lanes a - c) and mouse (lanes d - j) tau proteins. Tau polypeptides in total bovine brain extract (lane a), bovine microtubule protein (lane b), and thermal-stable, phosphocellulose-purified bovine tau protein (lane c) were compared. Lanes d - h contain 20 µg of 12 day fetus, 15 day fetus, 5 day newborn, 15 day newborn and adult brain proteins, respectively. Lanes i and j contain 20 µg of adult mouse cerebrum and cerebellum proteins, respectively.(Minor low molecular weight species here and in Figure 2 may be proteolytic products from tau since they appear below, but not above, the tau proteins).

Figure 2

Characterization of *in vitro* translated tau protein . Lanes 1 - 6 show immunoprecipitates of translation products using tau antiserum (lanes 1, 3 and 5) or nonimmune serum (lanes 2, 4 and 6). Translations were directed by beef brain mRNA (lanes 1 and 2), adult mouse brain mRNA (lanes 3 and 4) or 6 day old mouse brain mRNA (lanes 5 and 6). Lanes 7 - 10 show the efficient assembly into microtubules of the same translation products recognized by the tau antiserum. Lane 7: immunoprecipitate of tau from 6 day mRNA translation products. Lane 8: pellet after spinning microtubules made in the presence of translation products through sucrose. Lane 9: thermal-stable fraction of the microtubule pellet in lane 8 (equilvalent amount of translation products used for lanes 7 and 9). Lane 10: immunoprecipitate of tau from microtubule pellet (lane 8).

Figure 3

Purification of tau mRNA. Immunoprecipitates from translation products directed by total mouse brain mRNA (lanes 1 and 2), no added mRNA (lanes 3 and 4) or immunopurified tau mRNA (lanes 5 and 6). Nonimmune serum was used in lanes 1, 3 and 5. Lane 2 is the tau signal from 864,000 cpm of total brain mRNA translation products, while lane 6 is the tau signal from only 29,000 cpm of immunopurified tau mRNA.

Figure 4

Identification of tau cDNA by hybrid-selected translation. Lanes a and b are immunoprecipitates from 250,000 cpm of translation products directed by total 6 day brain mRNA using nonimmune serum (a) or tau antiserum (b). Lanes c - f are immunoprecipitates using tau antiserum on 60,000 cpm of translation products directed by mRNA bound to filters containing β-tubulin cDNA (lane c), pTA2 (lane d), pTA1 (lane e) or no DNA (lane f).

Figure 5

Developmental expression of tau mRNA. 1.5 µg of mRNA from 5, 10, 15, 20 day and adult mouse brain was subjected to gel electrophoresis on a 0.9% agarose gel containing formaldehyde and transferred to nitrocellulose. Tau mRNA was detected by hybridization to ^{32}P-labeled pTA2 probe. The faint 5 kb and 2 kb bands are ribosomal RNAs which sometimes show up as background. (Mouse ribosomal RNAs were used as molecular weight standards).

Figure 6

In vitro translation of tau protein from size fractionated 6 day mouse brain mRNA. Translation products directed by total mRNA were subjected to immunoprecipitation using nonimmune antiserum (PI) or tau antiserum (I). Total mRNA was fractionated on a 1% agarose gel containing methylmercury hydroxide. The 7.6 kb to 1.7 kb range of the gel was sliced into 10 pieces. Translation products directed by the eluted mRNA were subjected to immunoprecipitation with antiserum against bovine tau protein. The numbers above each lane represent the approximate logarithmic mean size of the translated mRNA. The faint bands seen at 2.8 kb and below in the immunoprecipitations are background due to α and β tubulin and actin which are heavily translated by mRNAs in this region. (Mouse ribosomal RNAs were used as molecular weight standards at 5 kb and 2 kb).

Figure 7

Genomic complexity and conservation of tau genes. (a) 20 µg of mouse genomic DNA was cut with PstI (lanes 1 and 4), Hind III (lanes 2 and 5) or Eco RI (lanes 3 and 6). Lanes 1 - 3 were hybridized to pTA1 probe while lanes 4 - 6 were hybridized to pTA2 probe. (b) 10 µg mouse DNA (lane 1), 10 µg human DNA (lane 2), 5 µg chicken DNA (lane 3), 5 µg frog DNA (lane 4), 4 µg fruit fly DNA (lane 5) or 2.5 µg yeast DNA (lane 6) was digested with EcoRI. The upper and lower blots were hybridized with pTA1 and pTA2 probes, respectively. (Molecular weights were determined with Hind III digested lambda DNA).

Figure 1

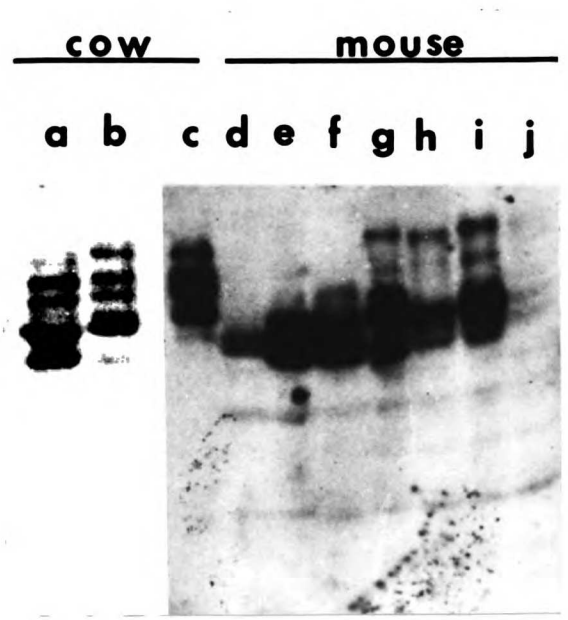


Figure 2

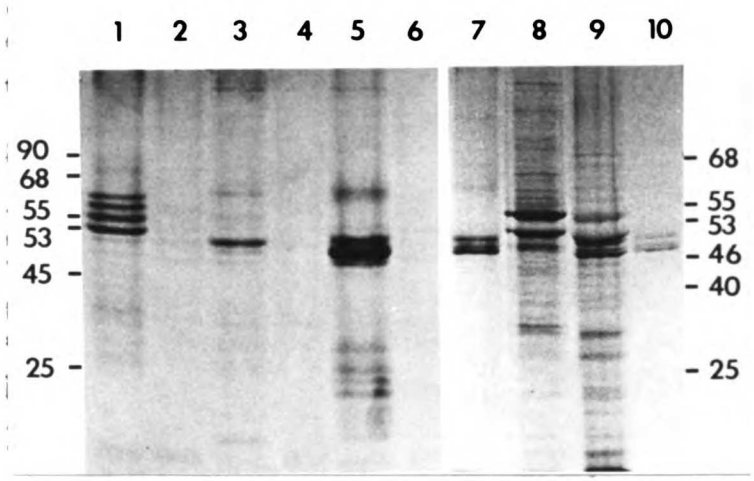


Figure 3

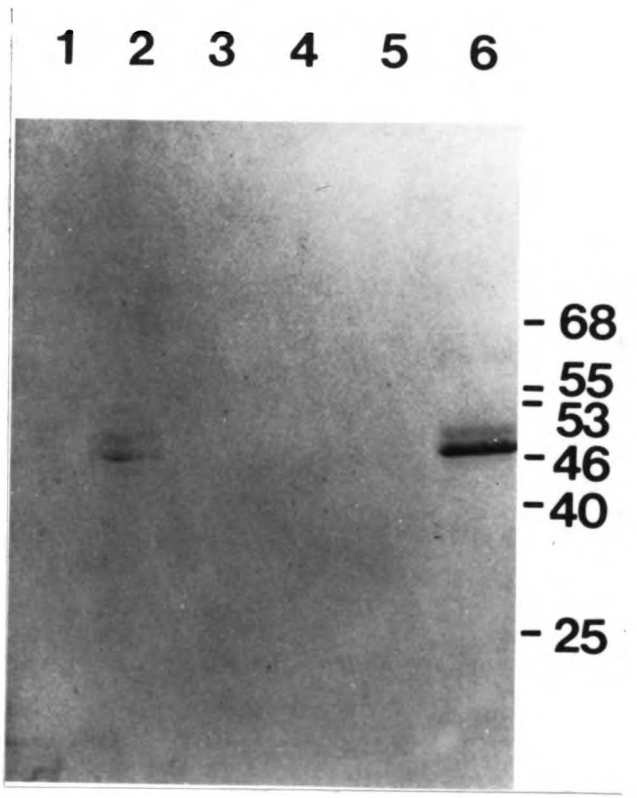


Figure 4

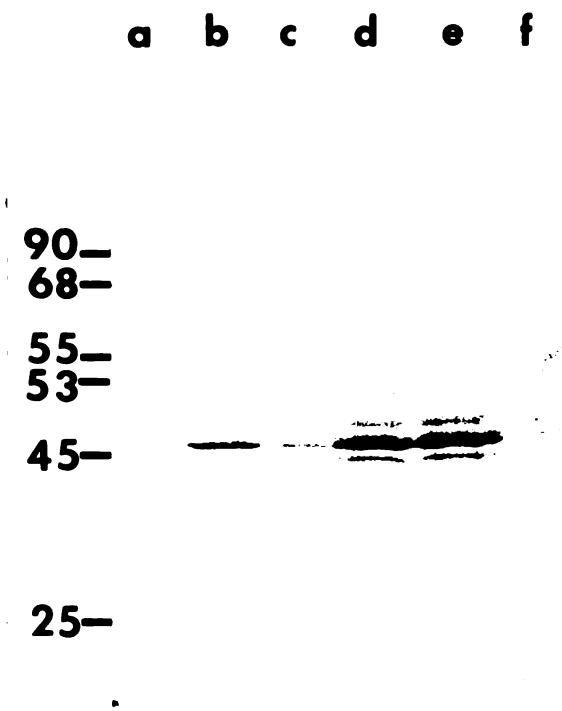


Figure 5

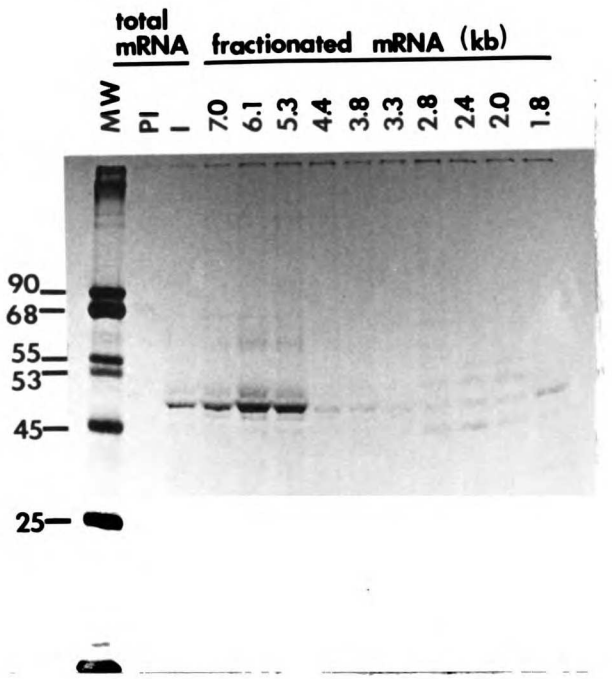

total mRNA
fractionated mRNA (kb)
MW
PI
I
7.0
6.1
5.3
4.4
3.8
3.3
2.8
2.4
2.0
1.8
90
68
55
53
45
25

Figure 6

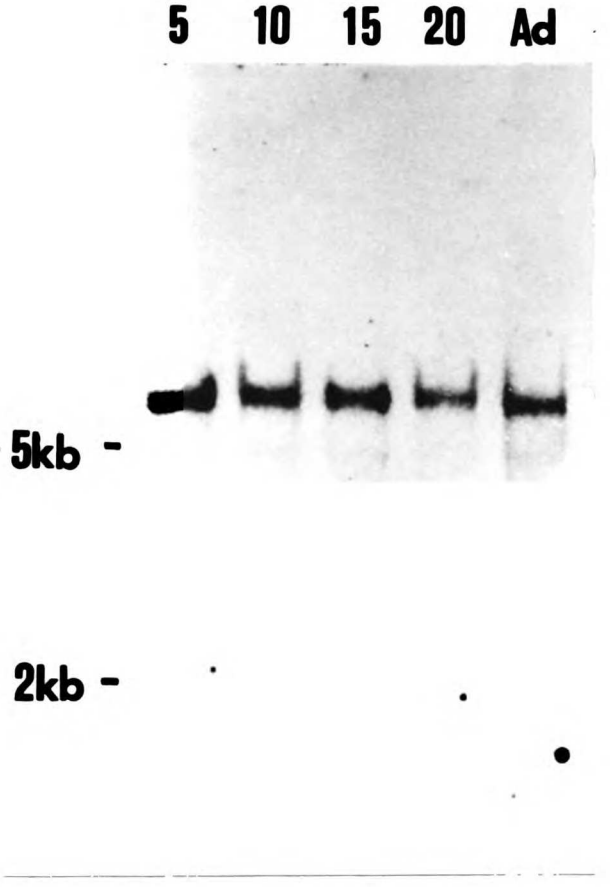


Figure 7a

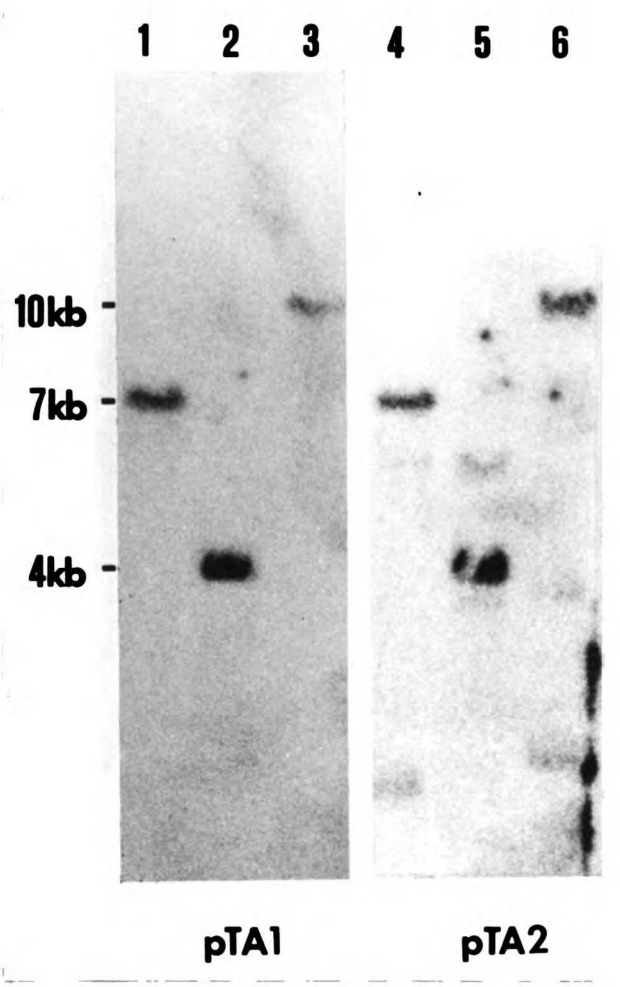
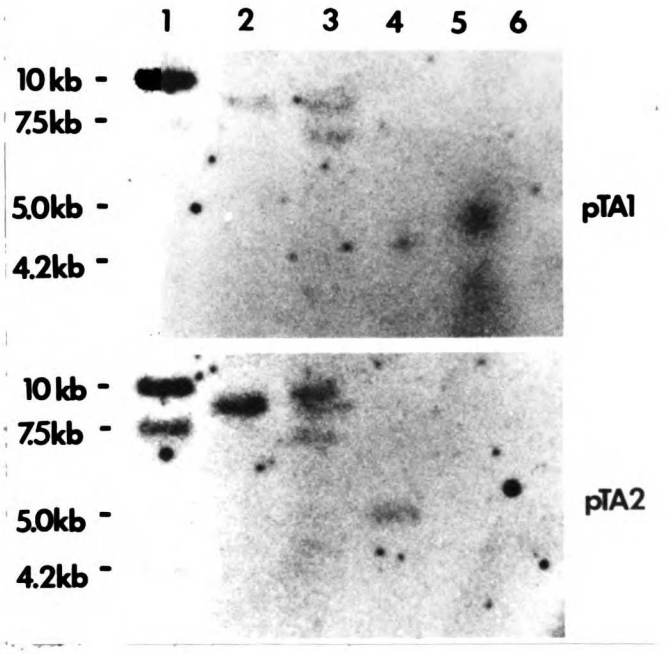


Figure 7b



CHAPTER THREE

Association of Tau Protein with Microtubules in Living Cells

INTRODUCTION

Several well-characterized microtubule-associated proteins (MAPs) coassemble with microtubules *in vitro* and promote the assembly of purified tubulin. These include the tau proteins of 55,000-68,000 kd (1,2), MAPs of 120,000 and 210,000 kd (3), and MAPs of approximately 300,000 kd, called MAP1 and MAP2 (4,5,6). The relevance of *in vitro* studies on MAPs rests first upon direct demonstration that the MAPs associate with microtubules in living cells, and second upon direct demonstration of their *in vivo* functions. Immunocytochemical studies have supported the conclusion that tau (7,8), the 210,000 kd MAP (9), and the 300,000 kd MAPs (8,10-14) bind microtubules in living cells. However, immmunocytochemical experiments alone can be ambiguous as evidenced by inconsistencies in reported staining patterns. For example, in our own studies with a highly specific affinity-purified antiserum raised against bovine brain tau protein we find vanishingly low levels of tau protein in cells previously reported to be stained by tau antibodies (see discussion below), and other investigators have noted discrepancies in high molecular weight MAP staining (12).

In this report, we re-examine the distribution of tau protein in established cell lines and in various mouse organs. We find that tau protein is found predominantly in cells of neuronal origin. We also use both biochemical and immunocytochemical techniques to demonstrate conclusively that tau protein is bound to microtubules in living cells. Finally, we discuss ways in which MAP function can be studied *in vivo*.

RESULTS

Tau distribution in mouse organs and established cells lines

Tau protein was originally characterized as 4-5 brain polypeptides of 55-60 kd which coassemble with purified tubulin and promote microtubule assembly *in vitro* (1). These polypeptides are closely related by physical properties and by primary structure (2). In addition, a microtubule-associated protein of 125 kd in rat pheochromocytoma and in mouse neuroblastoma cells was shown to be closely related to the 55-68 kd tau proteins by several criteria (15). In order to determine how widely tau protein is distributed outside of brain tissue where it was originally identified we performed immuno-blots (16) with a highly specific affinity-purified tau antiserum (17,18) on equal amounts of protein extracted from various mouse and rat tissues. The results of this study are summarized in Table 1. In some tissues the tau antibodies reacted with proteins of 42 kd of 200 kd. We have not characterized these proteins further but we note the results because the reaction with the tau antibodies appears to be highly specific.

The well-characterized tau proteins of 46-68 and 125 kd are most abundant in nervous tissue in general and in central nervous tissue in particular. The 125 kd tau protein first observed in established cell lines (15) is not an artificially produced protein unique to transformed cell lines, but is actually the major tau species in spinal cord, superior cervical ganglia, and dorsal root ganglia. In non-nervous tissue we detect only trace amounts of taus in the molecular weight ranges typical of well-characterized taus. We can not distinguish between the possibility that tau proteins are low in abundance in these

tissues and the possibility that our antibodies recognize neuron-specific antigenic determinants.

We have also examined tau distribution in established cell lines. The results of these analyses are summarized in Table 2. With the exception of HeLa cells, tau proteins are found only in cells of neuronal origin (PC12 cells display many features of nerve cells and derive from the neural crest (19)). The presence of tau in HeLa cells may be normal for epithelial cells or tau could be expressed inappropriately, as is vimentin (20), in some established cell lines. These results are inconsistent with earlier reports of tau distribution (7,8) based on immunofluorescence studies. The earlier studies found tau protein localized on microtubules in C6 glial and 3T3 fibroblast cells. When we use our affinity-purified tau antiserum which recognizes only tau proteins on immunoblots (18) containing total brain protein extracts, we do not detect any tau in C6 or 3T3 cells. Moreover, we have performed immunofluorescence studies on tau protein in 3T3 cells and several other fibroblast cell lines and detect no tau staining (see Tau Microinjection section below). These data do not exclude the possibility that tau protein is expressed in fibroblasts, but at levels that are undetectable with our affinity-purified tau antiserum. Indeed, tau protein was detected biochemically in 3T3 cells after coassembly of radio-labeled 3T3 proteins with brain tubulin (21,22). However, we now believe that tau protein is expressed predominantly in cells of neuronal origin. The trace amounts of tau detected in various non-neuronal tissues (Table 1) could be due to low levels of expression of tau in these tissues, or simply due to innervation of these tissues. We note

that tau protein has been unambiguously detected in one untransformed non-neuronal cell, the chicken erythrocyte (23).

Tau immunofluorescence

In order to study the subcellular localization of tau protein we performed immunofluorescence studies on glutaraldehyde-fixed cells with our affinity-purified tau antiserum. As mentioned above, in initial studies with mouse 3T3 cells we observed no tau staining. We therefore decided to study tau distribution in PC12 cells differentiated for 5 days in nerve growth factor because we had determined by immunoblotting that these cells contain high levels of tau protein (15). Figure 1, panel A shows a PC12 cell neurite stained with a mouse monoclonal α-tubulin antibody and a fluorescein-conjugated secondary antibody and visualized in the fluorescein channel. The neurite which is densely filled with microtubules (24) stains brightly and individual microtubule fibers can be seen in the growth cone where microtubules splay outward. The same neurite was stained with the rabbit affinity-purified tau antiserum and a rhodamine-conjugated secondary antibody (Figure 1, panel B). Notice that the neurite body stains brightly as with the tubulin antibody, and that the same microtubules are stained in the growth cone as are stained with the tubulin antibody (Figure 1, panel A). In control experiments, staining with tau or tubulin antibodies alone did not affect the staining pattern. Furthermore, the drug colchicine, which depolymerizes microtubules, abolished the tau staining pattern, and the drug vinblastine, which causes tubulin and tau to form paracrystalline aggregates *in vitro* (25), caused both the tubulin and tau staining to localize to elipsoid aggregates in the cytoplasm. In addition, cells which had low levels of tau protein as determined by immunoblotting were

devoid of tau immunofluorescence staining, showing that the immunofluorescence pattern is dependent on the presence of tau protein in the cell, and is not due to adventitious binding of tau antibodies to glutaraldehyde-fixed microtubules. We conclude that tau protein is bound to microtubules in differentiated PC12 cells. We have also determined by immunofluorescence that MAP1 is bound to microtubules in differentiated PC12 cells, consistent with findings for other cell types (14).

Microinjection of tau protein into rat fibroblasts

While immunofluorescence studies on tau distribution in PC12 cells demonstrated that tau protein is present on PC12 cell microtubules, the small size of PC12 cells and their round morphology make it difficult to determine if tau protein is localized exclusively on microtubules, particularly in the cell body. To address this question we microinjected purified tau protein into RAT1 fibroblast cells whose flat morphology is ideal for visualizing microtubules and other cellular organelles and looked at the tau localization by immunofluorescence. Figure 2, panel A, shows a field of RAT1 cells stained with the DNA-binding dye Hoechst. Normally these cells, like other fibroblasts we have examined, contain no detectable tau protein. The three cells marked with arrows were microinjected with 2 mg/ml tau protein, then the entire field was fixed with glutaraldehyde and stained with tau antiserum and rhodamine-conjugated secondary antibody. As shown in Figure 2, panel B it is clear that tau staining is seen only in the microinjected cells and that the staining is localized exclusively on the microtubules which can be seen to emanate from centrosomes. Thus, in living cells it appears that tau protein associates predominantly with microtubules and not with other organelles.

Biochemical demonstration of tau association with microtubules

While the immunofluorescence experiments described above strongly suggest that tau protein is associated exclusively with microtubules in living cells, the remote possibility exists that the glutaraldehyde-fixation employed causes tau protein to become artificially cross-linked to microtubules. To dispense with this possibility we sought a third criterion to demonstrate that tau is an *in vivo* microtubule-associated protein (MAP). One criterion used to identify MAPs independent of their *in vitro* properties is to prepare detergent-extracted cytoskeletons under microtubule-stabilizing conditions from normal cells, or cells treated with colchicine to selectively remove microtubules from their cytoskeletons, and to compare the non-tubulin proteins which selectively fractionate with tubulin (26). Figure 3 shows the results of such an analysis. An immunoblot was probed with our affinity-purified tau antiserum and an iodinated secondary antiserum. The first lane shows the tau protein found in a differentiated PC12 cytoskeleton. The third lane shows the extracted tau protein. Tau protein remains almost exclusively in the cytoskeleton fraction. However, if the microtubules are selectively depolymerized for 35 min with colchicine before lysis most of the tau protein is lost from the cytoskeleton fraction (second lane). The tau protein is now in the extracted fraction (fourth lane). These results shown biochemically that tau protein is associated almost exclusively with assembled microtubules in PC12 cells, and overcomes the concern that immunofluorescent localization of tau to microtubules is due to glutaraldehyde fixation artificially causing tau to associate with microtubules. We have

obtained similar results for MAP1 supporting earlier conclusions that MAP1 binds to microtubules in living cells.

Tau form dependent on interaction with microtubules

We have shown both biochemically and immunocytochemically that tau protein associates with microtubules in living cells. We would like to report one additional observation that perhaps demonstrates this association most strongly and also suggests a role for post-translational modifications in regulating the association of tau protein with microtubules. Using the extraction procedure described in the preceeding section, Pallas and Solomon showed that the extent of phosphorylation of some MAPs in vivo is dependent on whether they interact with microtubules (27). If the phosphorylation state of tau protein is dependent on its interaction with microtubules then treating cells with drugs affecting microtubule assembly should alter the phosphorylation state of tau protein. Since Lindwall and Cole showed that dephosphorylation of tau protein in vitro causes it to run with an increased mobility on SDS gels (28), it might then be possible to detect changes in tau phosphorylation, caused by microtubule drugs, on one dimensional gels. Figure 4 shows an immunoblot probed with our affinity-purified tau anti-serum. The first lane shows the tau pattern observed in differentiated PC12 cell extracts without drug treatment. Treatment with colchicine to depolymerize microtubules causes an increase in the mobility of all three tau species (second lane) similar to that observed upon treatment of purified tau with phosphatase (18). Taxol, which binds microtubules and shifts the tubulin monomer/polymer equilibrium to favor assembly, also causes this mobility shift (third lane). In addition, nocodazole which has the same affect on microtubule

assembly as colchicine, and vinblastine which causes tubulin and tau to co-aggregate, also cause the same mobility shift (not shown). Since these drugs which specifically affect microtubule assembly in different ways all cause tau mobility shifts when applied to living cells tau protein must interact directly with microtubules in cells. This result circumvents shortcomings of the approaches described in previous sections, where fixation or detergent-extraction might cause and adventitious association of tau with microtubules, since cells were lysed directly in SDS and boiled immediately. Although we have not formally demonstrated that the tau mobility shift is due to dephosphorylation we believe that this is the case since this mobility shift is exactly as described in vitro (28). Since tau protein form can be modified by changing the state of microtubules in a cell, it stands to reason that the cell may normally alter tau form to change the state of its microtubules.

DISCUSSION

We have examined the cellular distribution and subcellular localization of tau protein. As the result of an extensive analysis of tau levels in different tissues and different established cell lines we have come to view tau as a predominantly nervous-tissue-specific protein. In the course of these studies it became clear that the complexity of tau-related protein species is far greater than was originally described. Our highly specific affinity-purified tau antiserum recognizes polypeptides ranging from 42 kd to 200 kd in different tissues (Table 1). We have shown that the 125 kd tau observed in nervous tissues are closely related to the previously well-characterized taus (15). Further analysis will be required to prove

that other proteins that react with our tau antibodies are related to tau protein. The exact way in which the taus are related can only be determined by thorough analysis of tau cDNA clones. It is intriguing that we detect only one or a few tau genes in the mouse genome (18). The ability to translate multiple tau species from mouse brain mRNA in cell-free translation systems (18,29) suggests that a single tau gene may give rise to multiple tau mRNAs as has been seen for several genes now (see ref. 30 for example).

Since tau protein characterization was based on its in vitro association with microtubules it was important to demonstrate that the tau behaves like a MAP in living cells. We have demonstrated the association of tau with microtubules in cells in four ways. (1) Immunofluorescence using tau antibodies showed that tau protein binds microtubules in PC12 cells. Furthermore, all microtubules appear to be coated with tau protein. It remains possible that different tau subspecies react with different microtubules to specify functionally distinct microtubules. We will only be able to determine if this is the case if we can generate antibodies that distinguish between tau subspecies. (2) By microinjecting tau protein into fibroblast cells that normally lack tau protein we were able to verify that tau associates with microtubules in living cells. In addition, the flat morphology of the fibroblasts allowed us to determine that tau protein associates exclusively with microtubules in cells. (3) By preparing detergent-extracted cytoskeletons with or without microtubules we were able to show biochemically that tau is associated with the microtubule network. (4) Treatment of PC12 cells with any of four microtubule drugs

changes tau form showing that, independent of fixing or extracting cells, tau is still associated with microtubules.

While these experiments establish a strong set of criteria to demonstrate that a protein associates with microtubules in cells, new approaches must be found to determine the role of MAPs in cells. Ideally we might utilize a pharmacological agent or a mutation specific for tau protein. No drugs have been identified that specifically interfere with tau action. However, RAT1 fibroblast cells contain no detectable tau protein and are therefore analogous to genetic tau null mutants. We can introduce tau protein into these cells and we know that it will bind exclusively to microtubules (Figure 2). We plan to compare tubulin on and off rates in cells with or without tau by monitoring treatment, or recovery from treatment, with microtubule depolymerizing drugs. Microtubule dynamics can also be compared by introducing derivatized tubulin into cells (31) containing or not containing tau protein. We have extended *in vitro* studies on tau protein into living cells by demonstrating that tau associates with microtubules *in vivo*. The challenge for the future is to determine the *in vivo* function of tau protein.

ACKNOWLEDGMENTS

We thank David Gard for his assistance in mouse dissections. We are grateful to Stuart Feinstein for supplying PC12 cells and nerve growth factor, Steve Blose for supplying tubulin antibodies, and David Asai for supplying MAP1 antibodies and Cynthia Cunningham-Hernandez for preparation of the manuscript.

Table 1 Tau distribution in mouse and rat tissues

	Molecular weight of tau species			
Mouse tissue	42 kd	46-68 kd	125 kd	200 kd
cerebrum	-	+++	±	-
cerebellum	-	++	±	-
brain stem	-	++	±	-
spinal cord	-	++	++	-
7 day superior cervical ganglia (rat)	-	++	+	-
adult superior cervical ganglia (rat)	-	+	++	-
dorsal root ganglia (rat)	-	-	+	-
sciatic nerve (rat)	-	±	-	±
liver	-	-	-	-
spleen	++	±	-	-
thymus	++	±	-	-
heart	-	±	-	-
lung	-	±	-	-
pancreas	-	±	-	-
adrenal gland	-	-	-	+++
kidney	-	-	-	-
skeletal muscle	-	±	-	-
small intestine	-	±	-	-
lymph node	-	-	-	+++
esophagus	-	-	-	-
trachea	-	±	-	+

Key: -=not detectable, ±=trace, +=low, ++=intermediate, +++=high

Table 2: Tau distribution in established cell lines

	Molecular weight of tau species	
Cell line	55 - 68 kd	125 kd
Rat 6 glial	-	-
Mouse neuroblastoma N_2A	±	±
Mouse neuroblastoma N115 undifferentiated	±	±
Mouse neuroblastoma N115 differentiated	+	+++
Mouse 3T3 fibroblasts	-	-
Mouse BC_3HI brain tumor	-	-
Mouse NCTC L929 connective tissue	-	-
Mouse NCTC 1469 liver	-	-
Rat PC12 pheochromocytoma	+	+
Rat PC12 pheochromocytoma differentiated	+++	+++
Hamster CHO ovary	-	-
Human HeLa epithelium	+	-

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FIGURE LEGENDS

1. Tubulin and tau immunofluorescent staining of a PC12 cell neurite. Double-labeling was performed with monoclonal anti-αtubulin and fluorescein-conjugated goat anti-mouse IgG (a) and polyclonal rabbit anti-tau and rhodamine goat anti-rabbit IgG (b). Cells were washed in PBS, fixed (10 min in 0.3% glutaraldehyde, 1% Empigen BB (alkyl betaine, Allbright and Wilson, Whitehaven, Cumbria, England), 80 mM PIPES, 5 mM EGTA, 1 mM $MgCl_2$, pH 6.8), washed in PBS, incubated 7 min in 1mg/ml $NaBH_4$ in PBS, then washed 5x in PBS, 1% bovine serum albumin, 0.1% Tween 20. Primary antisera were incubated 30 min, washed as above, then secondary antisera were applied and washed as for primaries. Bar, 5 μm.

2. Microinjection of tau protein into rat fibroblast cells. (a) RAT1 cells stained with DNA-binding Hoechst dye. Cells marked with arrows were microinjected with 2 mg/ml tau protein in 90 mM KCl, 10 mM $NaPO_4$, pH 7.0. Forty minutes later the cells were fixed and treated for tau immunofluorescence as described in Figure 1 legend. (b) Tau immunofluorescence of same cells shown in (a). Bar, 15 μm.

3. Biochemical demonstration of tau association with PC12 cell microtubules. Detergent extracted cytoskeletons were prepared from differentiated PC12 cells as described in reference 26 and nonextracted or extracted tau proteins were analyzed by immunoblotting with tau antiserum. The left two lanes show tau proteins in PC12 cytoskeletons from untreated cells, or cells treated for 35 min with 10 μm colchicine to depolymerize the microtubules, respectively. The right two lanes show the extracted

tau proteins from nontreated and colchicine-treated PC12 cells respectively.

4. Effect of colchicine and taxol on tau form. Tau protein in extracts from PC12 cells treated with no drug, 10 μM colchicine for 6 hr, or 10 μM taxol for 6 hr was analyzed on an immunoblot probed with tau antiserum.

Figure 1

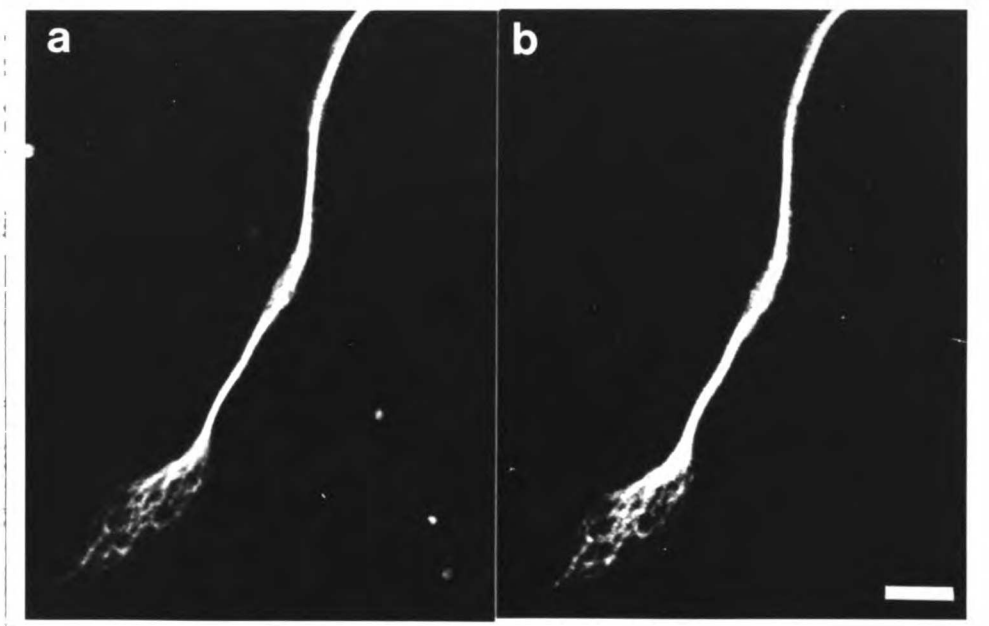


Figure 2

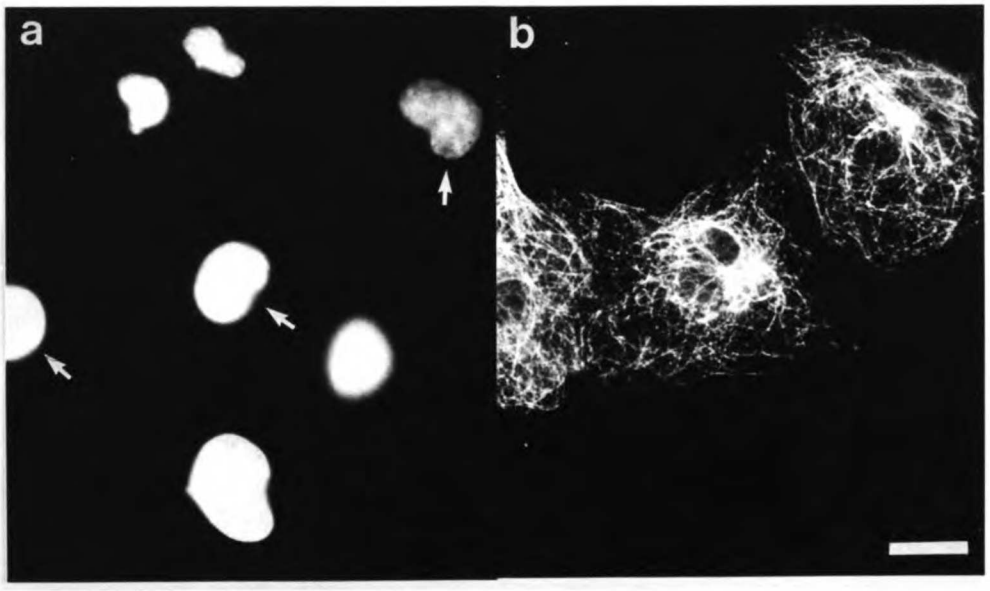


Figure 3

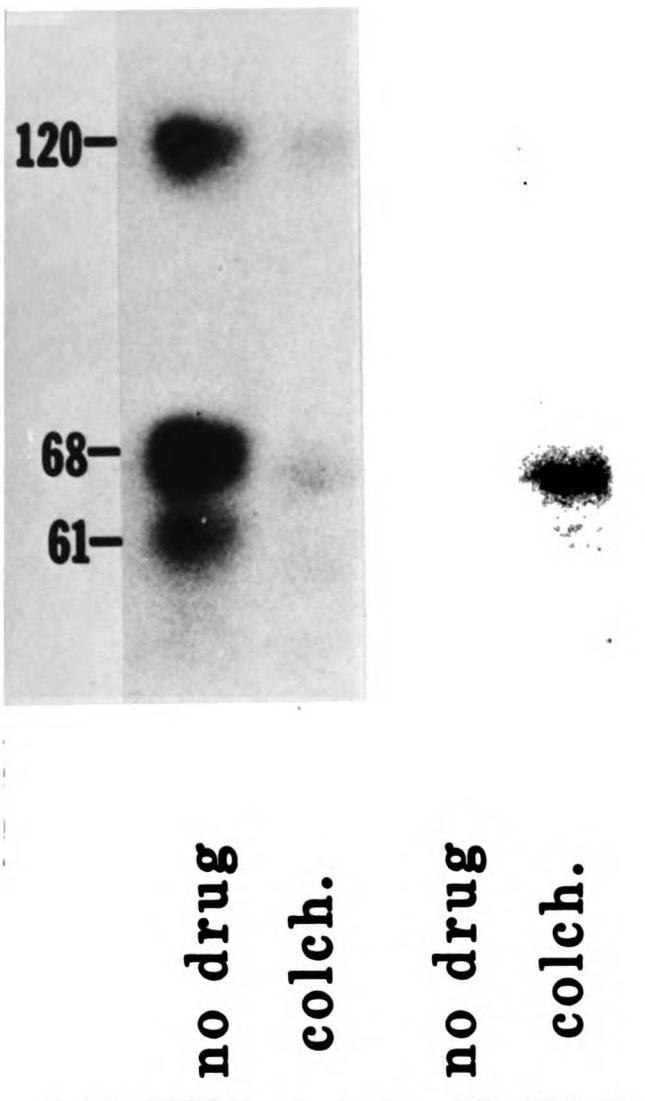
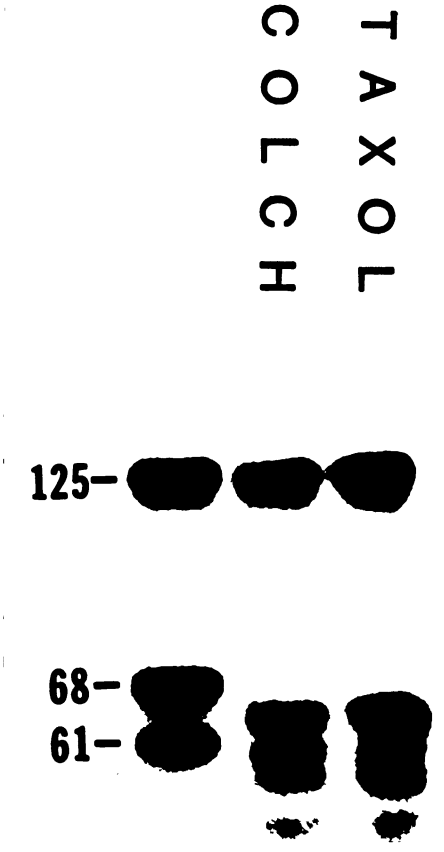


Figure 4



CHAPTER FOUR

Nerve Growth Factor-Induced Neurite Outgrowth in PC12 Cells Involves the Coordinate Induction of Microtubule Assembly and Assembly-Promoting Factors

ABSTRACT

Nerve growth factor (NGF) regulates the microtubule-dependent extension and maintenance of axons by some peripheral neurons. We show here that one effect of NGF is to promote microtubule assembly during neurite outgrowth in PC12 cells. Though NGF causes an increase in total tubulin levels, the formation of neurites and the assembly of microtubules follow a time course completely distinct from that of the tubulin induction. The increases in microtubule mass and neurite extension closely parallel 10- and 20-fold inductions of tau and MAP1, proteins shown previously to promote microtubule assembly *in vitro*. When NGF is removed from PC12 cells, neurites disappear, microtubule mass decreases, and both microtubule-associated proteins return to undifferentiated levels. These data suggest that the induction of tau and MAP1 in response to NGF promotes microtubule assembly and that these factors are therefore key regulators of neurite outgrowth.

INTRODUCTION

Extension of neuronal processes is fundamental to establishing the intricate wiring of the nervous system. These processes can bridge vast distances separating nerve cell bodies from their respective target cells. Electron microscopic analysis of neuronal processes has shown that they are densely packed with parallel arrays of filamentous structures, prominent among which are microtubules (35). Microtubules are present in all eukaryotic cells but are especially abundant in neurons where they play an essential role in both the extension and maintenance of neuronal processes (49, 39, 10), and additionally serve as tracks for vesicular transport between cell bodies and process tips (37, 47). Thus, in order to understand how these elongated neuronal processes are formed and how vesicular traffic is targeted within them, it is crucial to determine how the spatial and temporal arrangement of microtubule assembly is controlled. In particular, for further biochemical investigations we would first want to know if microtubule assembly in developing neuronal processes involves a rearrangement of pre-existing microtubules or the assembly of new microtubules.

Microtubule assembly has been studied extensively *in vitro* using neuronal tissue as a source of tubulin and associated proteins. Although pure tubulin monomers assemble poorly into microtubules (tubulin polymers) under physiological conditions, assembly is greatly stimulated by the addition of any of several protein cofactors that bind to microtubules. These cofactors include the tau proteins (48, 8), generally of 50-70kd, as well as microtubule-associated protein 1 (MAP1) and microtubule-associated protein 2 (MAP2) of approximately 300 kd (24, 32, 43). While the action of these cofactors has been studied

extensively in vitro, evidence that they promote microtubule assembly in vivo is still lacking. Several studies have shown that the overall activity of microtubule assembly-stimulating factors in brain and neuroblastoma extracts increases markedly during differentiation (30, 40). These findings are consistent with the notion that the extension of neuronal processes by differentiating nerve cells involves net microtubule assembly.

The study of microtubule assembly during neurite extension is facilitated by the use of PC12 cells, a clonal cell line derived from a rat pheochromocytoma (17). PC12 cells project long neurites in response to nerve growth factor (NGF), a target-derived protein which is a regulator of the development and maintenance of sympathetic and some sensory neurons (25, 42). PC12 cells are ideally suited for this study since neurite outgrowth is readily induced by NGF and the resulting neurites are filled with microtubules (29).

In this paper we have studied in detail the relationship between microtubule assembly and neurite outgrowth in PC12 cells. We find that increases in microtubule levels correlate precisely with increases in neurite length during NGF-induced PC12 cell differentiation. Furthermore, NGF causes total tubulin levels to increase, but the time course for this increase does not correlate with the increase in assembled microtubules, implying that an independent signal is responsible for promoting microtubule assembly. It is particularly significant that levels of two proteins shown previously to promote microtubule assembly in vitro and to co-localize with microtubules in vivo, tau and MAP1, increase with identical time courses to the induction of microtubule assembly and neurite outgrowth. These data

suggest that tau and MAP1 serve to promote microtubule assembly *in vivo* and are therefore key factors regulating neurite formation during neuronal differentiation.

MATERIALS AND METHODS

Cell Culture

PC12 cells were originally obtained from D. Schubert. They were grown in Dulbecco's Modified Eagle's Medium, supplemented with 10% "supplemented" calf serum and 5% horse serum (Hyclone Laboratories). Cells were cultured in a humidified 37°C incubator with a 12% CO_2 atmosphere. Prior to plating cells, culture dishes were treated with 100 µg/ml polylysine for 2 hr., then rinsed twice with sterile water and once with media without serum. Importantly, cells were plated at 1-2 x 10^4 cells per cm^2. The initial plating density can be a major source of variability between experiments. Plating at high density results in excessive cell clumping and severely limits the percent of cells that extend neurites as well as the density that the neurite network can attain. The extent of the neurite outgrowth response in turn affects the magnitude of the microtubule assembly induction and the microtubule protein level inductions. In addition, initial plating at high density causes tau and tubulin baseline levels to be elevated (see text), further limiting the full observed induction of these proteins:

When appropriate, NGF was added to 100 ng/ml approximately 12 hr after plating cells. The β subunit of 7S NGF was purified from mouse salivary glands by the procedure of Smith et al. (44). For some experiments dbcAMP was added to 1 mM. For priming experiments cells were grown in suspension on bacterial petri dishes (Falcon Labware, Oxnard, CA). Average neurite length was determined by measuring 200 neurites per day with a Numonics length digitizer (Numonics Corporation, Landsdate, PA), in random field photographs of PC12 cells cultured in NGF on three separate plates.

Tau, MAP1 and Tubulin Protein Level Determination

All protein extraction buffers contained 10 µM benzamidine HCl, 1 mM phenymethyl sulfonyl fluoride, 1 µg/ml 0-phenanthroline, 10 µg/ml aprotinin, 10 µg/ml leupeptin and 10 µg/ml pepstatin A to inhibit protein degradation. To prepare PC12 cell extracts, 100 mm culture dishes plated with cells were washed three times with 37°C phosphate-buffered saline (PBS : 0.13 M NaCl, 2 mM KCl, 8 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.2). Next, 0.5 ml of lysis buffer (LB: 25 mM Na_2HPO_4, 0.4 M NaCl, 0.5% SDS, pH 7.2) was applied to the plate. After 3-5 min. the viscous lysate was drained into a polypropylene microfuge tube and boiled for 3 min. The lysate was then centrifuged 10 min. in an Eppendorf centrifuge and the DNA-containing pellet was removed.

Protein determination was by the method of Lowry et al (28). 30 µl of extract was assayed in 1 ml reactions. Bovine serum albumin was used to generate standard curves and 30 µl of LB was added to each standard reaction to compensate for any interference introduced by constituents of LB in lysate samples.

SDS-PAGE was performed on 6% or 8.5% polyacrylamide gels (30:0.8 crosslinking) to resolve MAP1 or tau and tubulin, respectively (27). Separation of α and β tubulin was enhanced by raising the pH of the resolving gel to 9.2. Molecular weight standards were transferrin (90,000), bovine serum albumin (68,000) α tubulin (55,000), β tubulin (53,000), ovalbumin (45,000), chymotrypsin (25,000) and hemoglobin (15,500).

For tau and tubulin immunoblots, electrophoretic transfer was for 12 hr. at 50 mA using the conditions described by Burnette (6). Electrophoretic transfer for MAP1 immunoblotting was for 48 hr. at 30V

in 0.02% SDS, 0.15 M glycine. Microtubule proteins for mass standards were prepared from rat brain by the Weingarten et al (48) modification of the procedure of Shelanski et al (41). Rat brain microtubule protein concentration was determined by the procedure of Lowry et al (28), and relative concentrations of tubulin and MAP1 in the microtubule protein were estimated by densitometry of a Coomassie Blue-stained SDS-gel. For tau mass standards, heat stable microtubule protein was prepared from rat brain microtubule protein as described by Herzog and Weber (20). The protein concentration of the heat stable microtubule protein was determined by the procedure of Lowry et al (28) and the proportion of protein represented by tau was estimated by densitometry of a Coomassie Blue-stained SDS gel.

For quantitative immunoblotting, 30 µg per lane of PC12 cell lysates, and five different amounts of appropriate mass standards (10 ng - 2 µg tubulin, 10 ng - 1 µg MAP1, 1 ng - 200 ng tau), were loaded on SDS gels. Immunoblots were probed with either a MAP1 monoclonal antibody (kindly provided by David Asai) at 1:500 dilution, DMβ-1 and DMα-1 tubulin monoclonal antibodies (5) at 1:500 dilution, or affinity-purified tau antiserum (11, 36) at 1:200 dilution. Iodinated secondary antibodies, used at 10^6 cpm/ml, were prepared from the IgG fraction of goat anti-rabbit or anti-mouse IgG (Cappel Laboratories, Cochranville, PC) by the method of Hunter and Greenwood (21). Autoradiography was on Kodak X-omat AR film, using Dupont Lightening plus intensifying screens. Autoradiographs were aligned with immunoblots, and tubulin, tau and MAP1 levels quantitated by scintillation counting of nitrocellulose blot slices in Aquasol (New England Nuclear). For each set of samples analyzed by quantitative

immunoblotting, a duplicate set was subjected to SDS-PAGE and the resulting gel was stained with coomassie blue. Densitometry of occasionally underloaded or overloaded lanes, and of properly loaded lanes, provided a correction factor for tau, tubulin and MAP1 levels determined from parallel samples analyzed by quantitative immunoblotting. Underloading of samples was particularly problematic in the early parts of NGF time courses, where cell extract concentrations were significantly lower than for later samples, resulting in a systematic underestimation of protein levels in the Lowry assay. In addition, preliminary quantitation of autoradiographic data by densitometry proved inaccurate, presumably due to reciprocity failure (the lack of linearity between radioactive decay and autoradiographic grain density) of the x-ray film. Instead, quantitation by scintillation counting of immunoblot slices gave linear results over wide ranges of protein concentration. Together, the systematic error in the Lowry assay and the reciprocity failure during autoradiogaphy caused us to initially underestimate protein concentration early in NGF time courses, and therefore lead to overestimation of tau, tubulin, MAP1 and polymer level inductions.

Microtubule Mass Determination

To measure microtubule mass during PC12 cell neurite outgrowth, detergent-extracted cytoskeletons, free of unassembled tubulin, were prepared under microtubule-stabilizing conditions essentially as described by Solomon et al (45) The tubulin content of the cytoskeletons was measured following the scheme of Caron et al. (in preparation). Briefly, cells were washed once with 37°C PBS and once with extraction buffer (EB: 0.1 PIPES, 1 mM $MgSO_4$, 2 mM EGTA, 0.1 mM EDTA, 2 M glycerol,

pH 6.75). Cells on 100 mm plates were subsequently extracted twice for 8 min with 0.5 ml of EB containing 0.1% Triton X-100 and protease inhibitors (listed above). After draining excess EB from each plate, 0.5 ml of LB was added for 3-5 min. to solubilize the detergent-extracted cytoskeletons. In addition, the 1 ml of EB used to extract PC12 cells was centrifuged 1 min. in an Eppendorf centrifuge to collect insoluble material that came off the culture dish during extraction. This material was added back to the lysis mixture in LB. The viscous cytoskeletal lysate was boiled 3 min., then centrifuged 10 min in an Eppendorf centrifuge and the DNA-containing pellet was removed. The protein concentration of the extracted and cytoskeletal fractions was determined by Lowry assay. Equal amounts of cytoskeletal protein fraction samples were loaded on SDS-polyacrylamide gels and the tubulin contents were determined by quantitative immunoblotting, as described above, using rat brain tubulin as mass standards. The protein remaining in the cytoskeleton fraction reproducibly represented 25% of total cellular protein in undifferentiated PC12 cells, consistent with the finding of Solomon et al (45). Thus, the fraction of each cytoskeleton sample represented by polymeric tubulin was divided by four to determine the % of total cellular protein represented by polymeric tubulin. We found that during the course of neurite outgrowth the % of total protein which was not extracted by EB increased. We chose not to compensate for this increase in unextractable material in our calculations of % total protein in microtubules because the increase did not occur in a regular manner. Thus, the % of total protein represented by polymeric tubulin in Figure 2a may be underestimated by 10 - 25% for the later time points.

RESULTS

Neurite Outgrowth Involves Net Microtubule Assembly

As shown in Figure 1a, PC12 cells cultured in the absence of NGF have a rounded morphology. When these cells are cultured in the presence of NGF for several days, they acquire many characteristics of differentiated neurons (17). Most important for this study, neurites project from the cell bodies (Figure 1b). Additionally, maintenance of the neurite network is dependent upon the continued presence of NGF. NGF-differentiated PC12 cells withdrawn from NGF lose their neurites within 2 days (Figure 1c). Furthermore, as is the case with true neuronal processes, PC12 cell neurites contain many parallel arrays of microtubules (29). The growth and maintenance of PC12 cell neurites is dependent on these microtubules since treatment of differentiating (or differentiated) PC12 cells with colcemid for two to six hours to depolymerize the microtubules causes the neurites to retract or decay (Figure 1d and 1e). Nocodazole and vinblastine, which also disrupt microtubules, similarly cause neurite loss (data not shown).

To determine if PC12 cell neurite outgrowth involves net microtubule assembly or rearrangement of existing microtubules, the level of polymeric tubulin was measured in PC12 cells cultured for various times in the presence of NGF. Detergent-extracted cytoskeletons, containing only polymeric tubulin, were prepared under microtubule-stabilizing conditions. The tubulin content of these cytoskeletons was measured on immunoblots using defined amounts of purified rat brain tubulin as mass standards. During the time course of NGF administration, average neurite length was also determined. (Since substantial cell clumping occurred in PC12 cell cultures after 7 - 9

days of growth in NGF, we terminated all experiments in this study after approximately one week. We concentrated on early events in neurite outgrowth when cells were unclumped and actively extending neurites.) Microtubule mass, expressed as the percent of total cellular protein, and average neurite length are compared in Figure 2a. During the first two to three days in the presence of NGF little change is observed in microtubule mass and there is no appreciable neurite extension. After three days in the presence of NGF, however, microtubule mass and average neurite length increase progressively and in concert. Thus, neurite outgrowth is accompanied by net microtubule assembly, which from day 3 to day 9 of the experiment increases over two fold. Withdrawal of NGF from PC12 cells causes neurites to disappear and microtubule mass to return precisely to the starting levels (Figure 2a, arrows).

Several control experiments demonstrate that the extraction protocol employed cleanly and quantitatively separates assembled from unassembled tubulin. First, treatment of PC12 cells with colchicine for several hours to completely depolymerize microtubules (verified by immunofluorescence) resulted in virtually all tubulin becoming detergent-extractable. In addition, taxol treatment to drive microtubule assembly essentially to completion resulted in all tubulin becoming detergent-unextractable. Since the microtubule stabilizing conditions used in the extraction procedure are also conditions which favor microtubule assembly, one concern is that the extraction procedure might cause net microtubule assembly during extraction and therefore an overestimation of _in vivo_ tubulin polymer levels. However, Solomon et al (45) showed that under almost identical extraction conditions to ours, radiolabeled tubulin present in the extraction buffer does not become

incorporated into the cytoskeleton, and that the presence of GTP, which is required for assembly, does not affect tubulin retention. Finally, we found that increasing the glycerol concentration in the extraction buffer from 2 M to 4 M, which should further favor microtubule assembly, did not increase the proportion of tubulin in the detergent-extracted cytoskeletons.

Tubulin Accumulation Does Not Drive Microtubule Assembly

To test whether an accumulation of tubulin monomers drives microtubule assembly during neurite outgrowth, total tubulin levels in PC12 cells, cultured for various times in the presence of NGF, were analyzed by immunoblotting. As shown in Figure 3, tubulin levels increase slightly in response to NGF administration over a time course of 7 days (0-7), and decrease over 3 days of NGF withdrawal (-1,-2,-3). However, as can be seen in Figure 2b (open circles), quantitative analysis of this data clearly demonstrates that the time course of tubulin induction is completely distinct from that of the induction of microtubule assembly. Tubulin levels increase from day zero without any lag phase whereas microtubule levels remain unchanged during the first three days of NGF treatment, and then begin to increase. Since the tubulin induction precedes the microtubule assembly induction by several days, it is apparent that the percent of tubulin in the polymer form actually declines initially; it then rises several days later after new microtubule assembly is stimulated (Figure 2b, closed circles). These data suggest that tubulin accumulation does not drive microtubule assembly during neurite outgrowth. Rather, they suggest that a signal distinct from the accumulation of tubulin monomers must exist to promote microtubule assembly.

Tau and MAP1 Accumulate in Concert with the Induction of Microtubule Assembly

We next examined the question of whether microtubule assembly during neurite outgrowth might be induced by increasing levels of protein cofactors known to promote microtubule assembly in vitro. In earlier studies, two well-characterized microtubule-associated proteins identified in neuronal tissue, tau and MAP1, were found in PC12 cells (12, 16). A third well-known cofactor, MAP2, was not found. Employing biochemical and immunocytochemical criteria, we have previously shown that both tau and MAP1 are bound to microtubules in PC12 cells (12). In addition, it was previously shown by Greene et al. (16) that MAP1 levels are elevated in differentiated PC12 cells. We now examined the levels of tau and MAP1 in PC12 cells as a function of time cultured in the presence of NGF. Figure 4a shows an immunoblot probed with an affinity-purified antiserum raised against bovine brain tau protein. (This identical blot was subsequently probed with a tubulin antiserum to generate the autoradiogram shown in Figure 3. The faint bands seen above the tubulin bands in that figure are residual tau bands.) The three polypeptides recognized by this antiserum, migrating at 61, 68 and 125 kd, are coordinately induced in response to NGF treatment, and deinduced when NGF is withdrawn. Additionally, these proteins are closely related to one another in primary sequence as shown by peptide mapping (Drubin et al., in preparation). Figure 4b shows an immunoblot probed with a monoclonal MAP1 antibody. As with the tau proteins, MAP1 is induced in response to NGF treatment, and deinduced when NGF is withdrawn.

Tau and MAP1 data from several experiments are presented quantitatively in Figure 2c. During the first three days in the presence

of NGF, when little change in neurite length or microtubule mass is occurring (Figure 2a), tau and MAP1 levels remain unchanged at 0.025% and 0.04%, respectively, of the total cellular protein. After 3 days of exposure to NGF tau and MAP1 levels increase in concert with microtubule assembly and neurite outgrowth. By day 7, tau and MAP1 levels have increased to 10- and 20-fold above starting levels, and now represent 0.25% and 0.8% of the total protein, respectively. As discussed earlier, withdrawal of NGF from PC12 cells causes neurites to disappear and microtubule levels to decline within two days to the undifferentiated levels. Similarly, NGF withdrawal causes tau and MAP1 levels to rapidly return to their levels in undifferentiated cells (Figure 2c, arrow), with MAP1 levels decreasing slightly faster (compare Figure 4a with 4b). Thus, unlike tubulin levels, tau and MAP1 levels correlate closely with microtubule polymer levels both in the assembly and disassembly phases.

Microtubule Protein Accumulation in Response to Stimuli other than NGF

Several stimuli other than NGF can induce neurites, or can, in conjunction with NGF, affect the time course of neurite extension in PC12 cells. Having shown that neurite outgrowth involves net microtubule assembly, we sought to determine if factors that alter the rate or pattern of PC12 cell neurite outgrowth also affect tau, MAP1 and tubulin levels in a manner consistent with a model in which induced tau and MAP1 levels mediate net microtubule assembly. The first situation examined was growth of PC12 cells in dibutyryl cAMP (dbcAMP), an agent which induces PC12 neurites slightly faster than does NGF (18). As seen in Figure 5a, dbcAMP produces a more rapid induction of tau protein than does NGF. dbcAMP also induces MAP1 more rapidly than NGF, although in

this case to a lesser extent than is achieved by NGF (compare Figure 5b to Figure 4b). A second case studied by Gunning et al (18) is to administer both NGF and dbcAMP together. These agents act synergistically on PC12 neurite growth, causing neurites to grow much more quickly than with either agent alone. Treatment of PC12 cells with NGF plus dbcAMP causes both tau and MAP1 to be quickly and strongly induced (Figure 5a,b). Thus, stimuli that increase the rate of neurite outgrowth increase the rate of tau and MAP1 induction, consistent with the conclusion that these factors promote microtubule assembly during neurite outgrowth.

Neurite outgrowth in response to NGF can be prevented by denying PC12 cells access to an adhesive substrate. However, if PC12 cells are first grown in suspension in the presence of NGF and are then plated on an adhesive substrate, still in the presence of NGF, they now grow neurites without the lag phase shown in Figure 2a (15). This "priming" has been interpreted to result from the accumulation of limiting factors required to extend a neurite. Presumably, some of these factors may be those necessary to build new microtubules. Consistent with this idea, Greene et al. (16) found that MAP1 levels are elevated in primed PC12 cells. We concur with this result (Figure 6b) and also find that tau levels are elevated in primed PC12 cells (Figure 6a). Again, these results are consistent with the conclusion that tau and MAP1 support microtubule assembly in PC12 cells.

Finally, it has been found that crowding of PC12 cells can duplicate some of the biochemical changes caused by NGF, such as increasing choline acetyltransferase specific activity, but does not cause neurite

outgrowth (14). We found that cell crowding causes a tau protein induction (Figure 7), but no induction of MAP1 (not shown).

DISCUSSION

Neurite outgrowth involves net microtubule assembly

The mechanisms by which NGF regulates axon growth or regrowth and maintains the differentiated phenotype are not yet understood. For some time, however, it has been known that neuronal process extension and maintenance require intact microtubules. In fact, microtubules may be solely responsible for providing the force required to extend and maintain neuronal processes (31), while actin filaments may provide an opposing force in the neurite (46). One effect of NGF, then, would be to promote microtubule assembly in a growing process, and to maintain those microtubules in a differentiated process. Therefore, the first question we would want to address is: Does the formation of microtubules in a growing neuronal process involve a rearrangement of existing microtubules or the assembly of new ones?

We found that microtubule mass increases precisely in parallel with neurite extension (Figure 2a). This result is consistent with an earlier finding that differentiated neuroblastoma cells contain higher levels of assembled tubulin relative to undifferentiated cells (34). When NGF is withdrawn from differentiated PC12 cells, microtubule levels return to those seen in undifferentiated cells as neurites disappear. Thus, one effect of NGF is to stimulate net microtubule assembly as neurites are extended, and another is to maintain the increased levels of assembled microtubules.

Tubulin accumulation does not drive microtubule assembly

One possible mechanism to drive microtubule assembly during neurite outgrowth involves raising total tubulin levels in PC12 cells above the steady state level, thus driving tubulin polymerization. Net assembly should occur until the tubulin monomer concentration again reaches the steady state level. In this model, tubulin subunits are limiting for assembly.

We found that tubulin levels do indeed increase 2.5 fold in response to NGF (Figure 2b, open circles). However, the tubulin induction precedes the microtubule assembly induction by about 3 days (Figure 2a, open circles). Thus, tubulin does not appear to be limiting for net assembly. Furthermore, the data in Figure 2b (closed circles) indicate that, even when net microtubule levels first begin to increase, the percent of total tubulin in the polymeric form is still lower than in the undifferentiated state. This is because at first total tubulin levels increase faster than polymeric tubulin levels. Thus, tubulin is not limiting for assembly even after net assembly has been induced.

Several other investigators have also reached the conclusion that tubulin levels may not be limiting for microtubule assembly during neuronal process extension. First, tubulin levels in differentiating brain tissue or neuroblastoma cells do not change appreciably at the time of neurite extension (33, 39, 34, 13), even though process formation seems to involve net microtubule assembly (34, 13). Second, Heidemann et al. (19) investigated microtubule stability to anti-microtubule drugs in PC12 cells differentiated under conditions that increase the rate of neurite growth. They found that microtubules were most stable under conditions that produced the fastest neurite outgrowth. They speculated that the increased stability of fast-growing

neurite microtubules might result from increased levels of factors that favor microtubule assembly, and which normally limit the rate of neurite extension. The conclusion that tubulin levels are not limiting for microtubule assembly has also been reached by investigators studying non-neuronal systems. As frog oocytes develop into unfertilized eggs, and then become activated, a progressive and dramatic increase in the potential of the cytoplasm to support microtubule assembly occurs while tubulin concentration remains unchanged (23). It appears that these various cells have evolved strategies to regulate microtubule assembly that do not require drastic changes in total tubulin levels each time environmental signals dictate that changes in levels of assembled tubulin are needed.

The question therefore remains; What regulates microtubule assembly during neurite outgrowth? One possibility is that physiological conditions, such as GTP or calcium ion concentrations, could limit assembly. Alternatively, negative protein factors could inhibit assembly, or positive cofactors favoring assembly might be limiting. Changes in the activity of negative or positive regulators of assembly could be manifested by changes in their levels, or by changes in their form by post-translational modifications. Whatever the nature of the change is, the data presented here makes the strong prediction that it will occur in PC12 cells after three days of growth in the presence NGF, when microtubule levels begin to rise markedly.

Microtubule assembly-promoting factors are induced in parallel with the induction of microtubule assembly

The well-characterized microtubule-associated proteins identified in brain are good candidates for the limiting factors which drive

microtubule assembly during neurite outgrowth. They promote the otherwise poor assembly of tubulin in vitro, but evidence that they provide the same function in vivo is lacking. Consistent with the possibility that these factors promote microtubule assembly during neuronal process extension, extracts from differentiated neuroblastoma cells or developed brain tissue have significantly higher levels of microtubule-polymerization promoting activity than do the corresponding less-differentiated sources (30, 40). Also, many groups have reported that the microtubule-associated protein composition changes during brain development (30, 11, 3). We took advantage of the relatively uniform response of PC12 cells to NGF to analyze microtubule-associated protein levels during neurite outgrowth and microtubule level induction.

MAP1 has been identified in PC12 and N115 neuroblastoma cells and found to be present in higher levels after neurite outgrowth (16, 13). Also, we have previously identified and characterized tau protein in PC12 cells (12). In the present study we found that tau protein and MAP1 increase 10- and 20-fold, respectively, in response to 7 days of growth in the presence of NGF. The time course of neurite outgrowth, the induction of microtubule assembly, and increases in tau and MAP1 levels all occur exactly in parallel and show the same three day lag (Figure 2a,c). The induction of tubulin, on the other hand, occurs with no lag phase and does not correspond to the induction of microtubule assembly (Figure 2a,b). When NGF is withdrawn from differentiated PC12 cells, tau and MAP1 levels decline as microtubule polymer levels decline and neurites disappear. These results strongly suggest that tau and MAP1 are limiting factors for microtubule assembly during neurite outgrowth. In order to test this assertion directly we are presently trying to

alter tau and MAP1 levels in differentiating PC12 cells by microinjecting the cells with purified tau and MAP1 or antibodies raised against them.

Despite the 2.5 fold increase in tubulin level, the ratios of tau and MAP1 to total tubulin, and to assembled tubulin, increase dramatically during neurite outgrowth. Using 100kd, 350kd, and 85kd as the respective molecular weights of the tubulin dimer, MAP1, and PC12 tau (average of 125, 68, and 61kd), and the percent of total protein represented by each protein (Figure 2), we have calculated the approximate molar ratios of tau and MAP1 to total and assembled tubulin before NGF treatment and after full induction (Table 1). During neurite outgrowth ratios of tau to total tubulin increase from 1:70 to 1:17, while ratios of MAP1 to total tubulin increase from 1:200 to 1:25. These changes in tau and MAP1 stoichiometries with respect to tubulin are well within the effective ranges for promoting assembly determined in in vitro studies using physiological tubulin concentrations (7, 20, 26). We conclude that the inductions of tau and MAP1 we observe are well within the effective ranges for promoting microtubule assembly on the scale observed during neurite outgrowth.

Since we have previously shown that essentially all of the tau and MAP1 in PC12 cells is bound to microtubules (12), we can use the data in Figures 2a and 2c to estimate the molar ratios of these cofactors to assembled tubulin. Growth in NGF increases approximate tau to assembled tubulin ratios from 1:33 to 1:7 while approximate MAP1 ratios increase from 1:100 to 1:11. These values are close to saturation (7, 1). Since microtubules in differentiated PC12 cells are coated with such high levels of tau and MAP1, they should be markedly more stable than

microtubules in undifferentiated PC12 cells. This in fact has been observed (4, 19).

Microtubule protein accumulation in response to stimuli other than NGF

By examining the effects of culturing PC12 cells under conditions which alter the neurite outgrowth response, additional evidence has been obtained that tau and MAP1 are limiting factors during neurite extension. When the rate of neurite outgrowth is increased either in response to dbcAMP or to dbcAMP plus NGF, tau and MAP1 are induced more rapidly. Furthermore, both tau and MAP1 are induced in primed PC12 cells, and those cells show no lag phase when plated on an adhesive substrate (results summarized in Table 2). It should be noted that primed cells fail to project neurites when plated in the presence of NGF and cycloheximide (Stuart Feinstein, manuscript in preparation), suggesting that synthesis of new proteins in addition to tau and MAP1 is necessary for neurite outgrowth. Therefore, though we feel that tau and MAP1 are necessary for neurite outgrowth, they may not be sufficient to produce neurite outgrowth.

It is important to note that the induction of tau and MAP1 can be uncoupled. First, dbcAMP induces tau to a much greater extent than it induces MAP1. Neurites which grow in response to dbcAMP alone are shorter and less stable than those produced by NGF (18). Also, tau induction preceeds the MAP1 induction in cells treated simultaneously with NGF and dbcAMP. These data suggest that tau may be sufficient to promote the early phase of neurite outgrowth, but that MAP1 is essential for promoting microtubule stability and neurite elongation. In addition, cell crowding induces tau but not MAP1. The modulation of tau in this situation is of interest with respect to the work of Karsenti et al. (22) who found that the cytoplasm of crowded mouse L cells more readily supports microtubule assembly than does that of uncrowded L cells.

Perhaps the induction of tau in crowded PC12 cells is a manifestation of a general tendency of cells to favor higher levels of microtubule assembly when faced with crowding.

Finally, all of the stimuli tested cause a modest induction in PC12 tubulin levels (Table 2). The tubulin induction could result from the well-characterized autoregulatory control over tubulin synthesis (2, 9). Perhaps when net microtubule assembly occurs during neurite outgrowth, free tubulin levels diminish and, as a result, more tubulin is made. We have shown that this regulatory pathway can function in PC12 cells in response to colchicine (unpublished observation). However, while this mechanism may operate after 3 days of growth in the presence of NGF, it clearly is not the dominant mechanism prior to then. As shown in Figures 2a and 2b, tubulin levels increase during the first three days of growth in the presence of NGF, prior to the increase in microtubule assembly. Thus, we can say that a mechanism independent of the tubulin autoregulatory mechanism operates and in fact overrides the autoregulatory mechanism to increase total tubulin levels (despite elevated tubulin monomer levels) in anticipation of a need for more tubulin subunits to assemble microtubules in growing neurites. The coordinate synthesis and assembly of the major components of the microtubule cytoskeleton is essential for the morphogenesis of neuronal processes. Understanding how the synthesis of these components is regulated will be an important part of understanding how a neurite is assembled in response to nerve growth factor.

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Table 1: Approximate molar ratios of tau and MAP1 to total and assembled tubulin

	undifferentiated	differentiated
tau: total tubulin	1:70	1:17
MAP1: total tubulin	1:200	1:25
tau: assembled tubulin	1:33	1:7
MAP1: assembled tubulin	1:100	1:11

Table 2: Microtubule protein accumulation in response to various stimuli

	Neurite Growth	Tubulin Induction	Tau Induction	MAP1 Induction
NGF	normal	+	+++	+++
dbcAMP	fast	+	+++ (fast)	+ (fast)
NGF + dbcAMP	very fast	+	+++ (fast)	+++ (fast)
Suspension (+NGF)	-	+	++	+++
Crowding (-NGF)	-	+ (slow)	+ (slow)	-

- no response

+ slight induction

++ moderate induction

+++ large induction

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FIGURE LEGENDS

Figure 1: PC12 cell neurite outgrowth requires the continued presence of NGF and intact microtubules. (a) Undifferentiated PC12 cells. (b) PC12 cells cultured in 100 ng/ml NGF for 5 days. (c) PC12 cells cultured in 100 ng/ml NGF for 5 days, and subsequently withdrawn from NGF for the next 2 days. (d) PC12 cells cultured in 100 ng/ml NGF for 7 days, and subsequently treated with 10 µg/ml colcemid for 2 hours. (e) PC12 cells cultured in 100 ng/ml NGF for 7 days, and subsequently treated with 10 µg/ml colcemid for 6 hours. Bar in (a) represents 20 µm, same magnification used in (a-e).

Figure 2: Quantitative analysis of neurite length, microtubule mass, and microtubule protein levels during PC12 cell neurite extension. (a) Microtubule mass, determined by quantitative immunoblotting of 15 µg detergent-extracted cytoskeleton protein with anti-tubulin monoclonal antibodies ( ), and average neurite length for 200 neurites measured each day ( ), are plotted as a function of culture time in the presence of NGF. Arrows indicate data points collected after 2 days ( ) or three days ( ) of NGF withdrawal. (b) Total tubulin levels, determined by quantitative immunoblotting of 30 µg total PC12 cell protein with antitubulin monoclonal antibodies ( ), are plotted as a function of culture time in NGF. The arrow marks a data point collected after 3 days of NGF withdrawal. The % of total tubulin in polymer form ( ) was determined from the ratio of microtubule mass in 2a to total tubulin level in 2b. (c) MAP1 ( ) and tau ( ) protein levels, determined by quantitative immunoblotting of 30 µg total PC12 cell protein with a MAP1 monoclonal antibody and an affinity-purified anti-tau serum, are plotted

as a function of culture time in the presence of NGF. Arrows indicate data points collected after 3 days of NGF withdrawal.

Figure 3: Changes in total tubulin levels in response to NGF. Immunoblot of PC12 cell extracts probed with an anti-tubulin antiserum. PC12 cell extracts were prepared from cells cultured in NGF for 0 - 7 days. After 7 days, NGF was withdrawn from the cells and extracts were prepared after 1 - 3 days of withdrawal (-1,-2,-3). 30 µg of protein was loaded in each lane. Faint bands above tubulin bands explained in the legend to Figure 4.

Figure 4: Changes in tau and MAP1 levels in response to NGF. (A) The same blot shown in Figure 3 was probed here with an affinity-purified anti-tau antiserum. The faint bands seen above the tubulin bands in Figure 3 represent residual tau staining since the anti-tubulin antiserum was applied to the blot subsequent to the anti-tau antiserum. (B) Immunoblot probed with monoclonal MAP1 antiserum.

Figure 5: Tau and MAP1 induction by dbcAMP or NGF plus dbcAMP. Immunoblots of PC12 cell extracts probed with tau antiserum (A) or MAP1 monoclonal antiserum (B). PC12 cells were cultured in the presence of dbcAMP or NGF plus dbcAMP for 0 - 4 days.

Figure 6: Elevation of tau and MAP1 in "primed" PC12 cells. Immunoblots of PC12 cell extracts probed with tau antiserum (A) or MAP1 monoclonal antiserum (B). PC12 cells were cultured on bacterial petri dishes for 4

days in the absence (-) or presence (+) of 50 ng/ml NGF. PC12 cells do not extend neurites on petri dishes.

Figure 7: Induction of tau in response to cell crowding. Immunoblot of PC12 cell extracts probed with tau antiserum. Approximately 10^6 PC12 cells were plated on each 100 mm plate. Cells were cultured for 0 - 8 days in the absence of NGF. Under these conditions PC12 cells continue to proliferate and do not extend neurites.

Figure 1

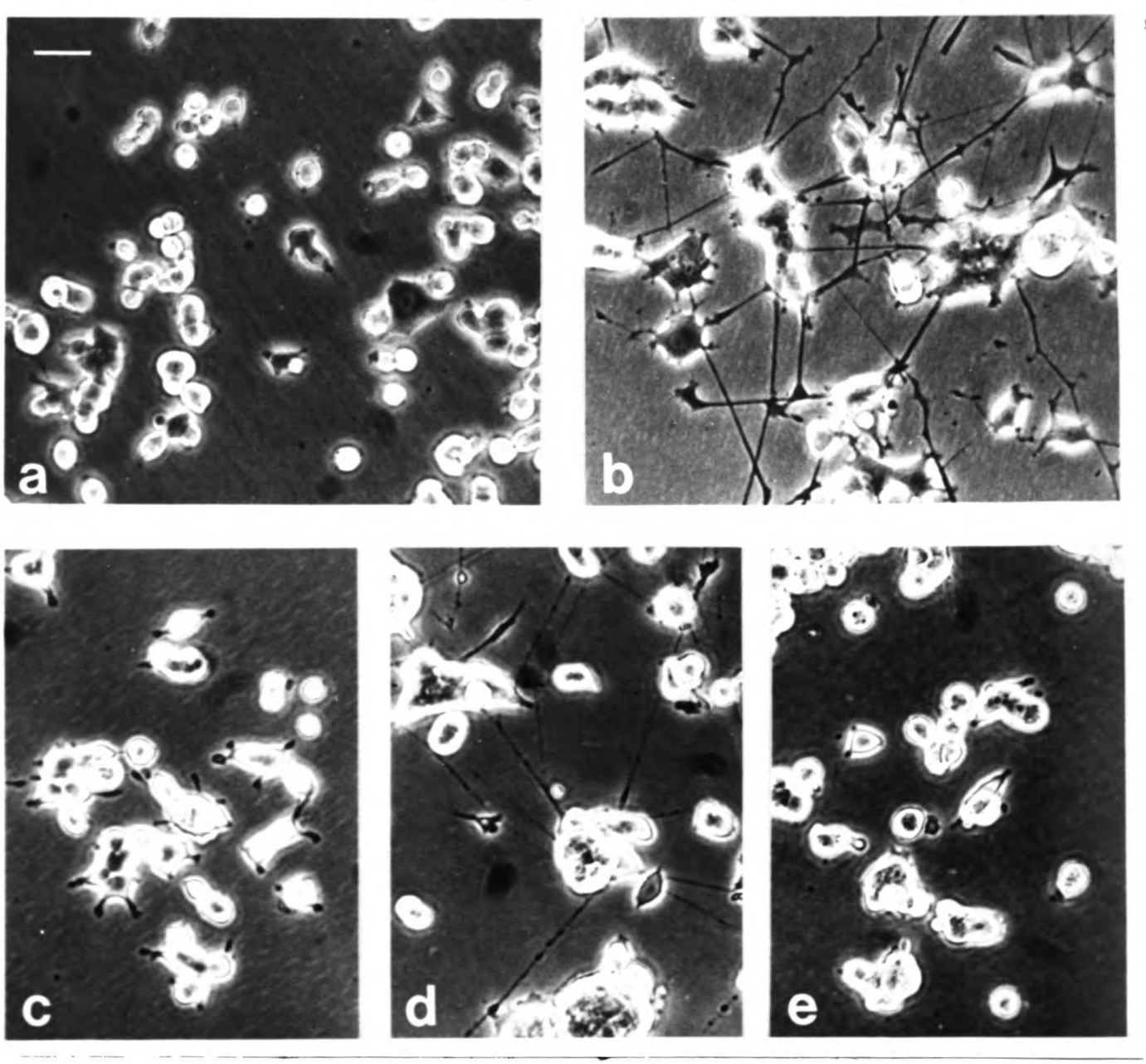

Figure 2

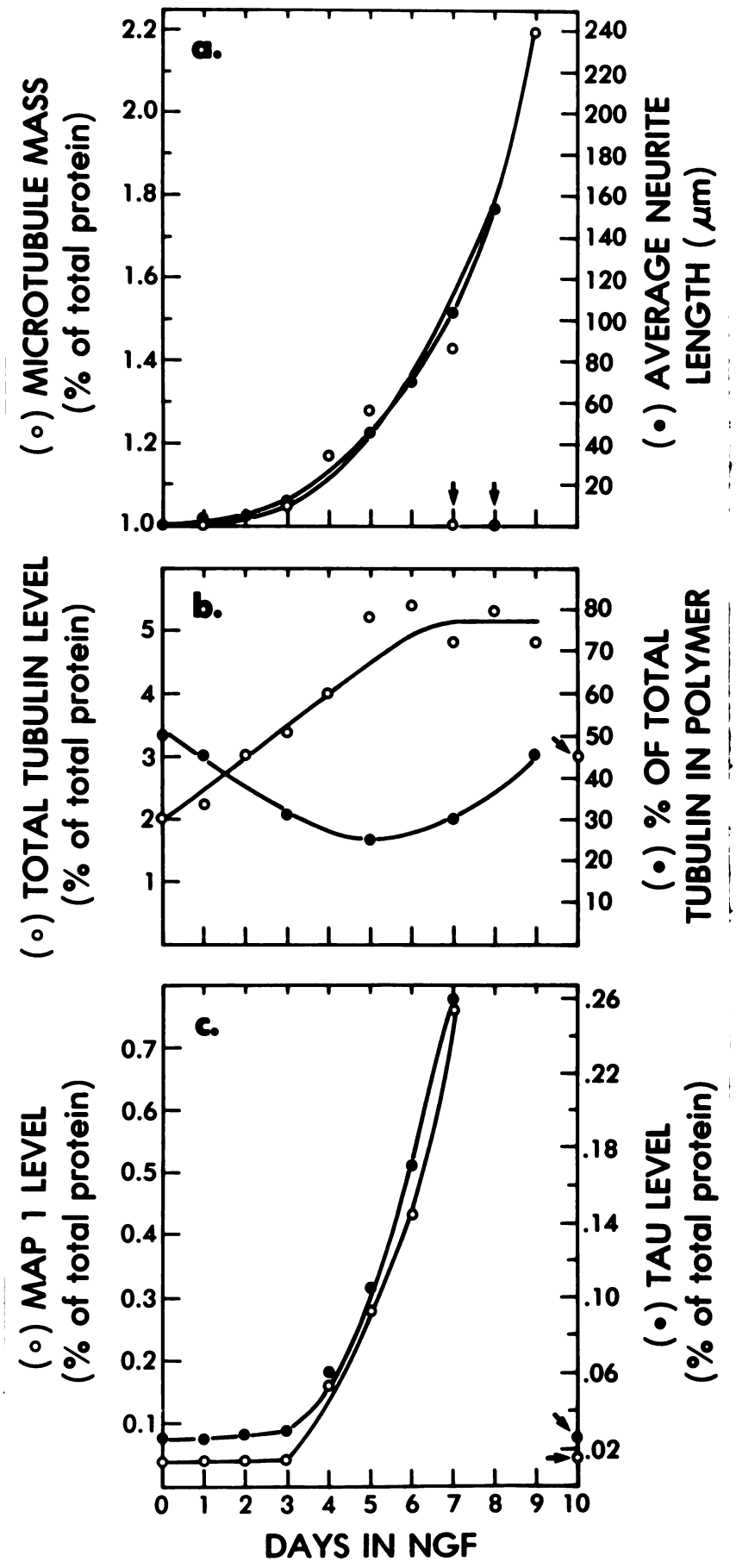
a.
(○) MICROTUBULE MASS (% of total protein)
(•) AVERAGE NEURITE LENGTH (μm)
2.2
2.0
1.8
1.6
1.4
1.2
1.0
240
220
200
180
160
140
120
100
80
60
40
20
b.
(○) TOTAL TUBULIN LEVEL (% of total protein)
(•) % OF TOTAL TUBULIN IN POLYMER
5
4
3
2
1
80
70
60
50
40
30
20
10
c.
(○) MAP 1 LEVEL (% of total protein)
(•) TAU LEVEL (% of total protein)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
.26
.22
.18
.14
.10
.06
.02
0 1 2 3 4 5 6 7 8 9 10
DAYS IN NGF

Figure 3

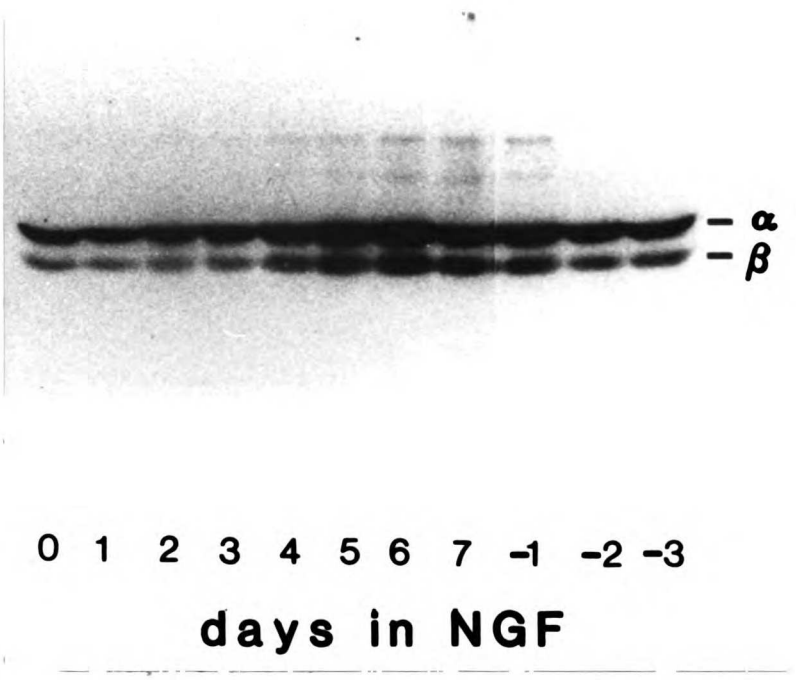

Figure 4a

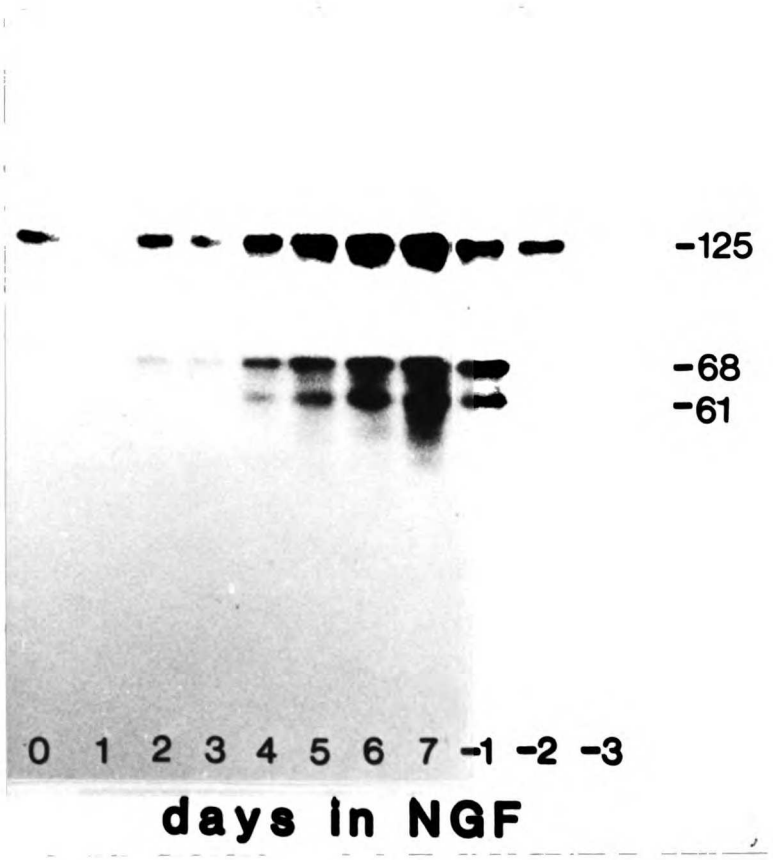

Figure 4b

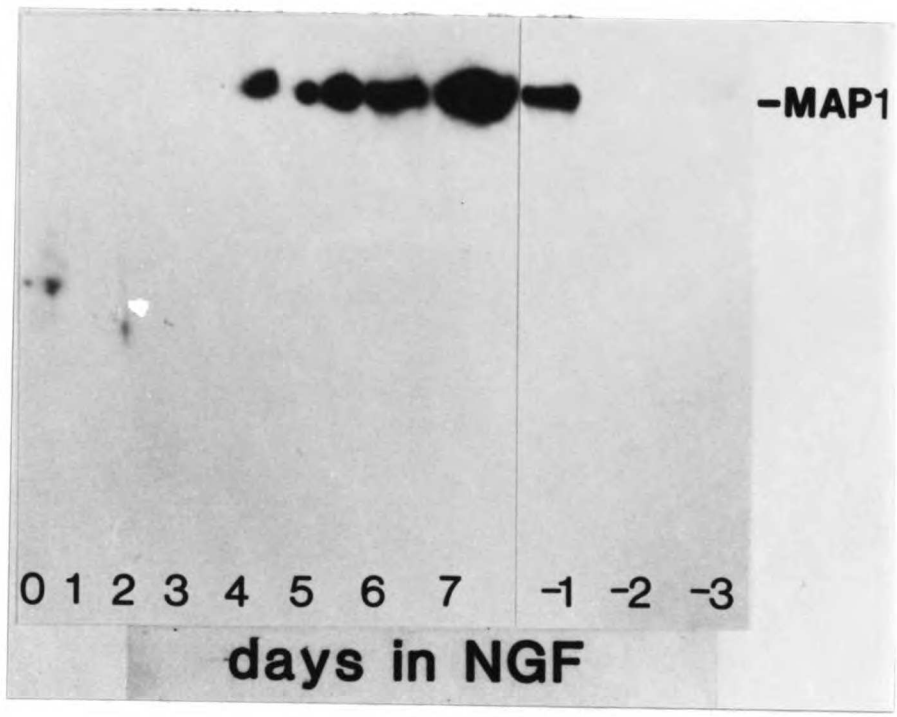

Figure 5

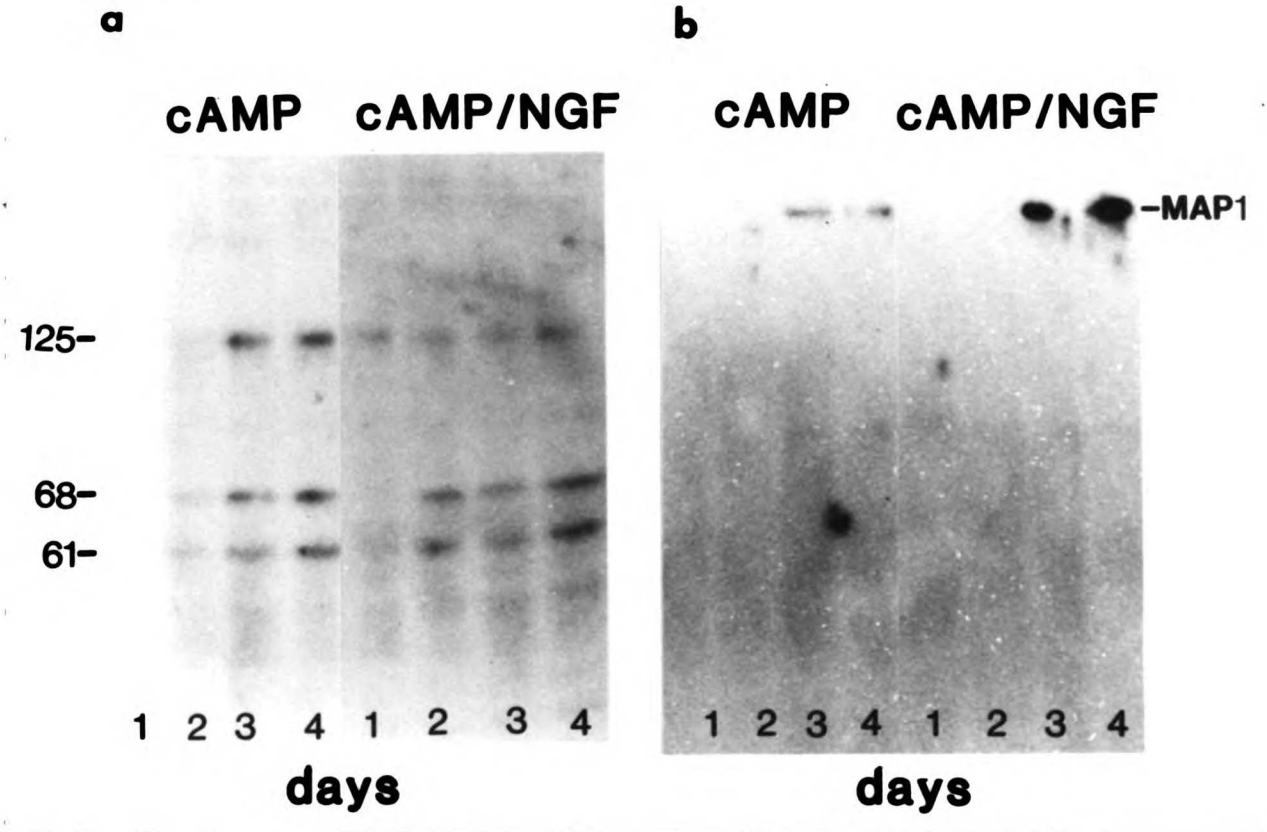
a
b
cAMP
cAMP/NGF
cAMP
cAMP/NGF
-MAP1
125-
68-
61-
1 2 3 4 1 2 3 4
1 2 3 4 1 2 3 4
days
days

Figure 6

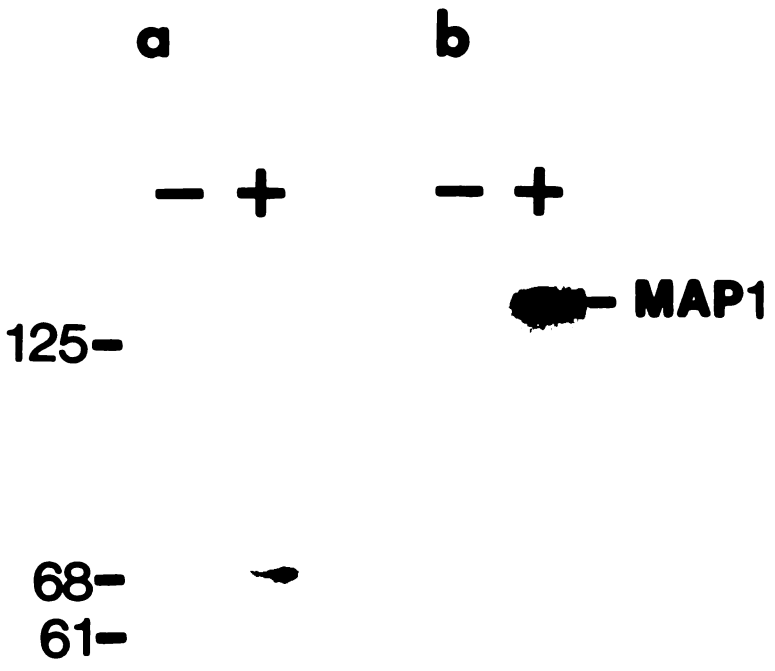

Figure 7