Differential Utilization of β-Tubulin Isotypes in Differentiating Neurites

Harish C. Joshi and Don W. Cleveland

Department of Biological Chemistry, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Abstract. β-Tubulin is encoded in vertebrate genomes by a family of six to seven functional genes that produce six different polypeptide isotypes. We now document that although rat PC-12 cells express five of these isotypes, only two (classes II and III) accumulate significantly as a consequence of nerve growth factor–stimulated neurite outgrowth. In contrast to previous efforts that have failed to detect in vivo distinctions among different β-tubulin isotypes, we demonstrate using immunoblotting with isotype-specific antibodies that three β-tubulin polypeptides (classes I, II, and IV) are used preferentially for assembly of neurite microtubules (with ~70% of types I and II assembled but only ~50% of type III in polymer). Immunofluorescence localization shows that an additional isotype (V) is partially excluded from neurites. Distinctions in in vivo localization of the neuron-specific, class III isotype have also been directly observed using immunofluorescence and immunogold electron microscopy. The sum of these efforts documents that some in vivo functional differences between tubulin isotypes do exist.

THE primary subunit of microtubules is the heterodimer of one α- and one β-tubulin polypeptide. In vertebrates, each of these polypeptides is encoded by a family of six to seven functional genes (reviewed by Cleveland, 1987) that produce five to six distinct polypeptide isotypes differing primarily in a ~15 amino acid carboxy-terminal variable domain (Sullivan and Cleveland, 1986). The observation that specific variable domain sequences have been highly conserved between species, despite their divergence within a species, has allowed classification of vertebrate β-tubulin genes into five isotypic classes (I–V; Sullivan and Cleveland, 1986; Lopata and Cleveland, 1987). The observation that specific isotypic sequences have been maintained during evolution suggests that isotypic differences have been selected through a functional constraint (as had been originally speculated by Fulton and Simpson, 1976; and Stephens, 1975).

Nevertheless, the use of genetic and biochemical approaches by a number of investigators has produced several lines of evidence that support the functional equivalence of β-tubulin isotypes. In particular, one β-tubulin has been shown to construct most classes of microtubules during spermatogenesis in *Drosophila* (Kemphues et al., 1982; Raff, 1984; Fuller et al., 1987). Further, a chimeric β-tubulin comprised of a chicken amino terminus fused to a yeast β-tubulin carboxy terminus has been shown to be incorporated into all classes of mouse fibroblast microtubules (Bond et al., 1986). Localization of the isotypes in different cultured fibroblast cells using indirect immunofluorescence has also demonstrated that all microtubules, at least at the resolution allowed by light microscopy, are copolymers of available isotypes (Lewis et al., 1987; Lopata and Cleveland, 1987; Gu et al., 1988). Even the most divergent β-tubulins, which are used to assemble marginal band microtubules in erythroid cells but whose sequences have not been conserved during evolution (Murphy et al., 1987), are coassembled into interphase and mitotic microtubules when inappropriately expressed in nonerythroid cells (Lewis et al., 1987; Joshi et al., 1987).

In fact, the only in vivo evidence yet to emerge for functional distinctions between isotypes are three examples of microtubules in specialized cells. In the first of these, not only were differences in in vitro assembly properties apparent for the major β-tubulin assembled into the marginal band microtubules of erythroid cells (Rothwell et al., 1986), microtubules enriched in this erythroid-specific isotype were found to be more stable to temperature-induced depolymerization (Joshi et al., 1987). However, the physiological relevance of such a distinction remains in question, a doubt encouraged by the finding that, alone among the vertebrate β-tubulins, the sequence of erythroid-specific subunits has not been conserved (Murphy et al., 1987; Cowan et al., 1988). In the second example, null mutations in the mec7 β-tubulin gene of *Caenorhabditis elegans* not only prevent assembly of the unusual 15 protofilament microtubules in touch cells, but also result in assembly of 11 protofilament microtubules. These 11 protofilament microtubules are not normally found in touch cells and are presumably assembled from the product of a different β-tubulin gene that alone cannot form 15 protofilament microtubules (Savage et al., 1989). In the final example, only one β-tubulin isoelectric variant is phosphorylated during neurite outgrowth (Edde et al., 1981; Gard and Kirschner, 1985).

To test further whether tubulin isotypes are used differen-

tially within animal cells, we have now focused on the microtubule arrays within neurons. This seemed a promising choice for four reasons. First, these cells possess specialized axonal microtubules which might be more likely to display isotypic distinctions (if they exist). Second, as mentioned earlier, it was known that a single isoelectric variant of β-tubulin is phosphorylated during neurite outgrowth. Third, although nothing is known concerning the biochemical differences, there was microscopic evidence that in lobster axons only a subpopulation (representing 25% of axonal microtubules) is used for the transport of vesicles through axoplasm (Miller et al., 1986). Fourth, two-dimensional reconstruction of serial thin sections had revealed biochemical heterogeneity in the microtubules in cultured neurites (Joshi et al., 1986). In this example, short segments of long neuritic microtubules were found to be resistant to cold-induced depolymerization.

To follow β-tubulin utilization during neurite outgrowth, we have now used rat PC-12 cells, a clonal cell line that extends neurites in response to nerve growth factor (NGF).[1] In the absence of NGF, PC-12 cells grow mitotically as round chromaffin-like cells, but in response to NGF they withdraw from the cell cycle and differentiate into sympathetic neuron-like cells (Greene and Tischler, 1976). In so doing, they transform their mitotic and cytoplasmic microtubule arrays into microtubules whose functions include neurite outgrowth, establishment and maintenance of asymmetric morphology (Yamada et al., 1970; Daniels, 1975; Edde et al., 1981; Landis, 1983; Greene and Tischler, 1982), and (possibly) fast axonal transport of vesicles through the cytoplasm. Unlike authentic sympathetic neurons, PC-12 cells have the further advantage that a pure population of cells can be obtained for biochemical analyses.

Our data demonstrate that three of the five β-tubulin isotypes expressed in these cells are induced during differentiation and that use of these isotypes is not random, but rather that some isotypes are preferentially polymerized into axonal microtubules. These findings demonstrate in vivo biochemical differences among the β-tubulin isotypes and support the hypothesis that different isotypes are preferred substrates for different microtubule-based processes in neuronal cells.

Materials and Methods

Cell Culture

PC-12 cells, a line derived from a rat pheochromocytoma (Green and Tischler, 1976), were grown on plastic dishes in DME supplemented with 5% bovine calf serum and 5% horse serum (Hyclone Laboratories, Logan, UT). For inducing differentiation, cells were triturated from the surface and plated (3×10^4 cells/100-mm plate) onto tissue culture dishes (Falcon Labware, Oxnard, CA) that had previously been treated with 1.0 mg/ml poly-L-lysine for 30 min then rinsed twice with water. 100 ng/ml of NGF (Sigma Chemical Co., St. Louis, MO) was added to the medium. This NGF-containing medium was changed every 48 h.

Rat sensory neurons were cultured using a procedure slightly modified from Fields et al. (1978). Dorsal root ganglia (DRG) were dissected from the exposed vertebral column of a newborn Sprague–Dawley rat by slitting the vertebrae at the dorsal midline and gently removing the ganglia (with their attached spinal nerve roots) from the lumbosacral region with fine forceps. The ganglia were placed in Liebovitz's L-15 medium (L-15 obtained from Gibco Laboratories, Grand Island, NY; supplemented with 0.6% glucose, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin), rinsed twice, and then treated with 0.25% trypsin for 20 min at 37°C. The trypsin was removed, L-15 plus 10% fetal calf serum was added, and ganglia were triturated mechanically by forcing them through a 19-gauge needle to obtain a cell suspension. After filtration through cheese cloth to remove large tissue fragments, the cells were rinsed once and resuspended in L-15 containing 0.6% methyl cellulose and 100 ng/ml NGF. Cells were plated onto polylysine-coated glass coverslips lying in a 100-mm culture dish (10^4 cells/dish). The low plating density facilitated unambiguous identification of neurons from fibroblasts and Schwann cells. Cultures were maintained at 37°C in a humidified incubator.

1. *Abbreviations used in this paper*: C/S, cytoskeletal-to-soluble protein ratio; DRG, dorsal root ganglia; NGF, nerve growth factor.

Antibodies Used

Rabbit polyclonal antibodies specific to five vertebrate β-tubulin isotypic classes I–V (originally described by Lopata and Cleveland, 1987) were used in this investigation. In brief, the antibodies were raised against 9–15 amino acid peptides corresponding to consensus sequences for the carboxy-terminal domains of vertebrate β-tubulin isotypes I–V. All antibodies used here, except type III, were affinity purified by chromatography of each antiserum on an affinity column produced by covalently linking the respective carboxy-terminal peptide to Sepharose. Precise conditions of affinity purification and detailed characterization of these antisera are given in Lopata and Cleveland (1987). A polyclonal antibody specific for tau polypeptides was a kind gift from Drs. M. Kirschner and D. Dreschel (University of California, San Francisco, CA). MAP-2 monoclonal antibodies, AP21 and AP18, were very generously provided by Dr. L. Binder (University of Alabama, University, AL). α- and β-Tubulin monoclonal antibodies (DM1A and DM1B) were obtained from Amersham Corp. (Arlington Heights, IL).

Production of Cloned Fusion Proteins for Each Isotypic Class of β-Tubulin

Plasmids used for producing fusion proteins (comprised of an amino-terminal 32 kD of the bacterial protein trpE linked to the β-tubulin isotype sequence beginning at residue 345 and continuing through to the normal carboxy terminus) were as described earlier (Lopata and Cleveland, 1987). For isotypes I–IV, plasmids producing the carboxy-terminal sequences of the mammalian isotypic variants were used; for type V, the chicken carboxy-terminal sequence was used.

To induce fusion protein accumulation in bacteria, overnight cultures harboring the respective plasmids were diluted (1:10) into M9 media without tryptophane and grown for 2 h at 30°C with vigorous aeration. Indole acrylic acid (solubilized in ethanol) was added to 5 μg/ml to induce expression of the *trp* operon and the cultures were allowed to grow 4 h at 30°C. Extracts containing the fusion proteins were prepared by pelleting the bacteria by centrifugation, washing the cell pellets once with 10 mM Tris-HCl (pH 8), and resuspension in gel sample buffer (Laemmli, 1970). After boiling for 5 min, bacterial DNA was pelleted by centrifugation (5 min at 12,000 *g*) and the supernatant containing solubilized proteins was recovered. Samples were electrophoresed immediately or stored at −20°C.

Quantitative Analysis of β-Tubulin Isotypes in PC-12 Cells

To determine accurately the amount of each β-tubulin isotype present in various samples, each was electrophoresed on a series of parallel gels along with serial dilutions of a bacterial extract containing one of the cloned fusion protein isotypes. Each gel was blotted and processed with the antipeptide antibody that recognized the corresponding fusion protein, followed by ^{125}I-protein A to detect the presence of bound primary antibody. Autoradiography of the fusion protein dilutions provided internal standards for densitometric quantitation.

The amount of cloned fusion protein contained in a specific lysate dilution was obtained by coelectrophoresis of a known volume of fusion protein extract with serial dilutions of known amounts of bovine albumin. From densitometric comparison of the intensity of the Coomassie blue staining of the fusion protein and the albumin standards, the amount of fusion protein was determined. Knowing the molecular weight of the fusion protein, the molar amount of β-tubulin carboxy terminus in a specified volume of lysate was then calculated. The absolute accuracy of this method relies upon the assumption that the fusion proteins bind Coomassie blue with the same efficiency as does albumin. Even if this is incorrect, the relative amounts of the various isotypes will still be measured correctly since the fusion pro-

teins are nearly identical in amino acid composition (and must bind Coomassie blue to the same extent).

The autoradiographic image of each blot lane was quantified using a soft laser densitometer (Zenieh, Fullerton, CA) and compared against a standard curve of the integrated optical densities of the fusion protein dilutions. For determination of soluble and cytoskeletal levels of each isotype, only points where both soluble and cytoskeletal values fell within the linear range of the analysis were used.

Dilutions of fusion protein standards were concurrently blotted in all experiments to insure the accuracy of the resultant quantitation.

Preparation of Cell Extracts Containing Soluble, Polymeric, and Total Tubulin

To prepare extracts of total cell proteins, a 100-mm tissue culture dish containing 1×10^4 cells/cm^2 was washed twice with 5 ml of PBS at 37°C and then lysed with 500 μl of 25 mM Tris (pH 6.8), 0.5% SDS. The lysate was boiled (in a microfuge tube) to lower the viscosity of DNA and protein concentrations were determined by bicinchoninic acid assay (Smith et al., 1985). To prepare the soluble and polymeric fractions of cell tubulin, cells were washed twice very gently with a microtubule stabilizing buffer (0.1 M *N*-morpholinoethanesulfonic acid, pH 6.75, 1 mM $MgSO_4$, 2 mM EGTA, 0.1 mM EDTA, 4 M glycerol). Soluble proteins were extracted at 37°C for 4–6 min in 500 μl of microtubule stabilizing buffer containing 0.1% Triton X-100. The soluble extract was removed and centrifuged for 2 min in order to pellet any cytoskeletal material dislodged from the culture dish during extraction. The remaining cytoskeletal fraction in the culture dish was dissolved in 500 μl of 25 mM Tris (pH 6.8), 0.5% SDS, and combined with the pellet from the soluble extraction. Protein concentration was again determined by the bicinchonic acid assay (Smith et al., 1985). Before gel electrophoresis, all the extracts were brought to 1× gel sample buffer (Laemmli, 1970).

Gel Electrophoresis and Immunoblotting

Polyacrylamide gels for analysis of protein samples were performed as described (Laemmli, 1970). Proteins were electrophoretically transferred to nitrocellulose (BA 85; Schleicher & Schuell, Inc., Keene, NH) in one-half strength Laemmli gel running buffer containing 20% methanol. Typically, transfer was for 4 h at 50 V. Filters containing transferred samples were stained with Ponceau S (0.2% Ponceau S in 3% trichloroacetic acid) to identify the positions of molecular weight standards. Nonspecific protein binding sites were blocked by incubation in 4% bovine albumin in phosphate-buffered Triton X-100 (0.2% Triton X-100, 0.15 M NaCl, 10 mM sodium phosphate [pH 7.5], 1 mM EGTA) for 10 min. For immunological detection, primary antibody in phosphate-buffered Triton X-100 containing 4% albumin was allowed to react overnight at room temperature. Unbound primary antibody was removed and the filters were washed five times (3 min each wash) in G buffer (0.5% Triton X-100, 50 mM triethanolamine [pH 7.4], 0.1 M NaCl, 0.1 mM EDTA, 1 mM SDS). ^{125}I-Labeled protein A was added and incubated for 1 h at room temperature, followed by five washes (3 min each wash) in G buffer to remove unbound protein A. Binding was detected by autoradiography using DuPont Co. (Wilmington, DE) Lightning Plus intensifying screens and Eastman Kodak Co.(Rochester, NY) XAR film.

Indirect Immunofluorescence

For the visualization of individual tubulin isoforms in the microtubules of differentiated PC-12 cells and primary cultures of rat dorsal root ganglion neurons, glass coverslips containing cells were washed for 15 s at 37°C with stabilization buffer (0.1 M *N*-morpholinoethanesulfonic acid [pH 6.9], 1 mM EGTA, and 4 M glycerol [Solomon et al., 1979; Osborn and Weber, 1982]) and then incubated for 1 min at 37°C in stabilization buffer containing 0.5% Triton X-100. Coverslips were then rinsed in stabilization buffer (15 s at 37°C) and plunged into methanol at −20°C for 5 min. Coverslips were rehydrated in PBS and stained either with the isotype-specific antibody (diluted 1:20 in PBS) or a β-tubulin monoclonal antibody for 1 h at room temperature. They were then rinsed with PBS and a fluorescein-labeled anti-rabbit IgG antibody (Cooper Biomedical, Inc., Malvern, PA) was applied for 45 min at room temperature. The coverslips were rinsed with PBS and mounted in aquamount (Lerner Laboratories, New Haven, CT).

The cells were examined using conventional light microscopy on an Olympus Corp. (New Hyde Park, NY) BH2 microscope with epi-fluorescence optics and photographed on Kodak Tri-X film developed in Diafine. Alternatively, for higher resolution, cells were examined using confocal scanning laser microscopy (White et al., 1987).

Quantitative Immunofluorescence Microscopy

For detecting the relative quantities of tubulin isotypes in the microtubules of PC-12 cell bodies and their neurites, we have used a ratio imaging procedure using a fluorescence digitizing system constructed by D. Lansing Taylor (Carnegie Mellon University, Pittsburgh, PA). Briefly, lysed and fixed cells were incubated successively with an isotype-specific rabbit polyclonal antibody, a mouse monoclonal IgG that recognizes all β-tubulin isotypes, a rhodamine-conjugated sheep anti-rabbit IgG, and a fluorescein-conjugated sheep anti-mouse IgG. Images in both rhodamine and fluorescein channels were digitized using a charged coupled device, and analyzed for the ratio of fluorescein to rhodamine intensities "pixel-by-pixel" with an on-line computer.

Immunogold Electron Microscopy

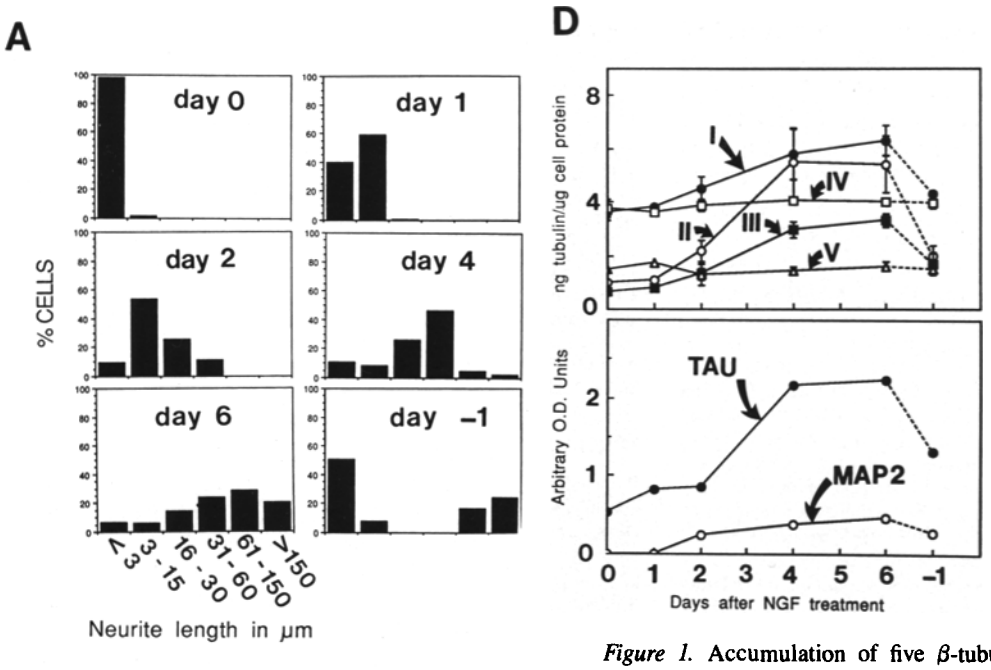
For localization of each tubulin isotype on individual microtubules of PC-12 neurites, we used the procedure of Geuens et al. (1986). Briefly, cells grown on glass coverslips were fixed and permeabilized simultaneously for 1 min with 0.5% gluteraldehyde and 1.0% Triton X-100 in buffer I (Hanks buffered salt solution containing 5 mM Pipes, 2 mM $MgCl_2$, and 2 mM EGTA, pH 6). Cells were subsequently taken through the following treatments: 0.5% glutaraldehyde in buffer I for 10 min; 0.5% Triton X-100 in buffer I for 30 min; 0.5 mg/ml $NaBH_4$ in buffer I for 15 min; TBS (10 mM Tris, 140 mM NaCl, pH 7.6) for 5 min. Cells were incubated at room temperature for 8–10 h (overnight) with the primary antibodies in TBS. Cells were washed four times, 5 min each with TBS and incubated for 6 h with 1/2 dilution of 5 nM colloidal gold-labeled secondary antibodies (Janssen Life Sciences Products, Piscataway, NJ). Cells were washed again four times, 5 min each with TBS and postfixed with 1% gluteraldehyde for 10 min, 2% OsO_4 for 30 min, impregnated with 0.5% uranyl acetate for 30 min, dehydrated with an ethanol series, embedded in Polybed 812 (Ladd Research Industries, Burlington, VT), and ultrathin (gray-to-silver) sections were examined using an electron microscope (Carl Ziess Inc., Thornwood, NY).

Results

Quantitation of Individual β-Tubulin Isotypes, Tau Polypeptides, and MAP-2 during Differentiation of PC-12 Cells

To examine tubulin isotype utilization in PC-12 cells, we initially sought to determine which β-tubulin isotypes are expressed during NGF-induced neurite outgrowth and the level of accumulation of each isotype during differentiation. Within 1 d after addition of NGF, the cells cease dividing and extend growth cones. These neurites continue elongating and by the end of 6–7 d in NGF, most cells develop an extensive network of long, anastomosing neurites. A histogram of the length of extended neurites is presented in Fig. 1 *A*. Extension requires the continuous presence of NGF inasmuch as withdrawal results in regression of neurites and clumping of the cell bodies (Fig. 1 *A*, *day −1*).

To determine the levels of individual β-tubulin isotypes, we performed quantitative immunoblot analysis with each of five isotype-specific polyclonal antibodies. The antibodies were generated by use of synthetic peptides antigens (Lopata and Cleveland, 1987) representing the consensus carboxy-terminal amino acid sequences from five conserved β-tubulin isotypic classes (Sullivan and Cleveland, 1986; Lopata and Cleveland, 1987). Quantitative standards for each isotype were obtained by preparing known amounts of bacterially derived fusion proteins comprised of the bacterial protein trpE linked to the ~100 carboxy-terminal amino acids of each β-tubulin isotype. A gel of the bacterial extracts con-



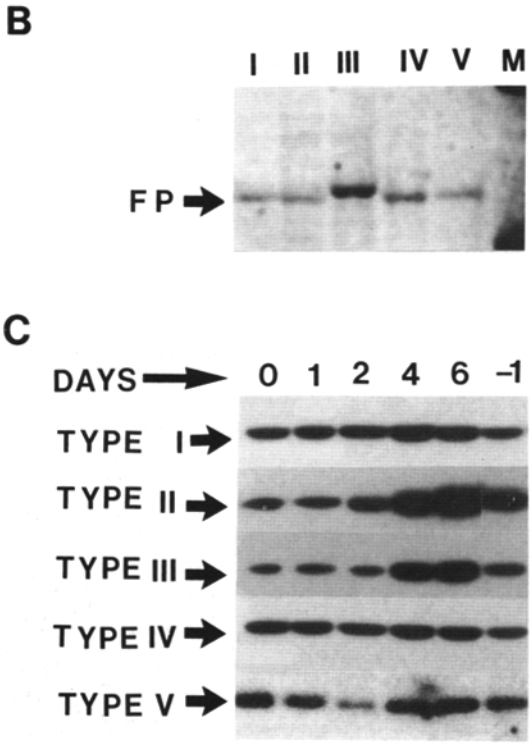


Figure 1. Accumulation of five β-tubulin isotypes during NGF-induced differentiation of PC-12 cells. (*A*) Histogram displaying the length distribution of neurites extended from PC-12 cells after addition of NGF. Lengths of 100–200 neurites were measured from five randomly chosen fields of NGF-stimulated PC-12 cultures. (The length of branched neurites is defined to be the sum of the lengths of all the branches of one neurite.) Day −1 represents 6 d of growth in NGF followed by a 1 d of withdrawal from NGF. (*B*) Preparation of known amounts of bacterially derived, isotype-specific fusion proteins. Coomassie blue-stained polyacrylamide gel of bacterial lysates containing the fusion proteins (*FP*) used to quantify each β-tubulin isotype. The arrow points to the position of the fusion proteins which are comprised of an amino-terminal portion from trpE linked to the carboxy-terminal sequences of each β-tubulin isotype. Lane *M* contains molecular mass markers (45 and 66 kD). (*C*) Immunoblot analysis of isotype accumulation during neurite outgrowth. 50 μg of total cell protein from PC-12 cells that had previously been treated with 100 ng/ml NGF for 0, 1, 2, 4, and 6 d or 6 d followed by 1 d in the absence of NGF (*−1*) were electrophoresed on five parallel gels. Autoradiograms are of immunoblots with type I, II, III, IV, and V antibodies, respectively. (*D*) Quantitation of accumulation of β-tubulin isotypes I–V, tau polypeptides, and MAP-2 during neurite extension in PC-12 cells. Densitometric quantitation of three independent experiments measuring β-tubulin isotype accumulation (like that shown in *C* is displayed as a function of the number of days of culturing in NGF. All quantitative measurements used concurrent immunoblotting of fusion protein dilutions to produce internal quantitation standards.

taining fusion proteins for isotypes I–V is shown in Fig. 1 *B*. In each case the fusion protein accumulates as one of the major proteins in the bacterial extract, with negligible contamination with background proteins. Hence, we were able to determine the amount of fusion protein present by comparison with Coomassie blue staining of known amounts of albumin electrophoresed in adjacent lanes.

Extracts were prepared from parallel aliquots of PC-12 cells that had been incubated with 100 ng/ml NGF for 0, 1, 2, 4, or 6 d and also from a culture that had been treated for 6 d with NGF and then withdrawn for 1 d (Fig. 1 *A*, *day −1*). The protein concentration in each extract was determined and equal amounts of protein from each extract were blotted in adjacent lanes of eight parallel polyacrylamide gels. Appropriate dilutions of each fusion protein were also electrophoresed and blotted. Autoradiograms of five immunoblots probed with each of the five isotype-specific antibodies are shown in Fig. 1 *C*.

Densitometry was used to quantify the amount of each isotype, using each fusion protein dilution series as standards to confirm that all measurements were in the sensitive range of the immunoblot assay and to generate accurate standards

for measurement of the absolute amount of each isotype. The outcome of the experiment in Fig. 1 *C* and three additional independent experiments are plotted in Fig. 1 *D*. Although total tubulin levels were increased only about twofold during differentiation (in agreement with earlier reports; Drubin et al., 1985), individual isotypes accumulated to markedly different extents. Most apparent was the induction of types II and III, which increased significantly (four- to fivefold), while isotype I increased modestly (about twofold) and isotypes IV and V remained almost unchanged. These differences alter the quantitative contribution of isotype II from 9% of total β-tubulin in undifferentiated cells to 26% in differentiated cells, while isotype III goes from 6% to 16%. Although we found accumulation of isotype I to be somewhat variable in four independent experiments (but always within a factor of two), isotypes II and III were invariably induced at least four- to fivefold.

We also examined whether the preferential accumulation of isotypes II and III during differentiation is coincident with the increase in the levels of the microtubule-associated proteins, MAP-2 and tau polypeptides. Autoradiograms of immunoblots using either a polyclonal antibody that is specific for tau (kindly provided by Drs. D. Dreschel and M. Kirschner) or monoclonal antibodies that recognize MAP-2 (AP21 and AP18, from Dr. L. Binder) were also scanned by densitometry. These quantitative data, along with the corresponding data for β-tubulin, are presented in Fig. 1 *D*. Clearly, both tau and MAP-2 accumulate in a pattern not markedly different from the increase in tubulin isotypes. Total levels of tau polypeptides increase four- to fivefold (in agreement with earlier analyses; Drubin et al., 1985, 1988) and that of MAP-2 increase from undetectable to a significant level.

Microtubule Assembly during Neurite Outgrowth Correlates Temporally with Tubulin Accumulation

From the quantitative analyses of tubulin isotypes induced during neurite outgrowth, we draw two conclusions. First, as a result of selective accumulation of isotypes I, II, and III, the composition of the β-tubulin pool is constantly altered as the cells acquire a neuronal phenotype in response to NGF. Second, isotypes II and III accumulate concomitantly with the increase in the levels of tau polypeptides and MAP-2. To test whether these changes in the isotype composition temporally correlate with neurite outgrowth or alternatively whether tubulin synthesis precedes outgrowth (as has been reported by Drubin et al., 1985), we made soluble and cytoskeletal extracts (Solomon et al., 1979; Osborn and Weber, 1982) of PC-12 cells at 1-d intervals after addition of NGF. Equivalent proportions of each fraction were analyzed by quantitative immunoblotting with a monoclonal antibody that recognizes all isotypes equally (Lopata and Cleveland, 1987; Lopata, M. A., and D. W. Cleveland, unpublished ob-

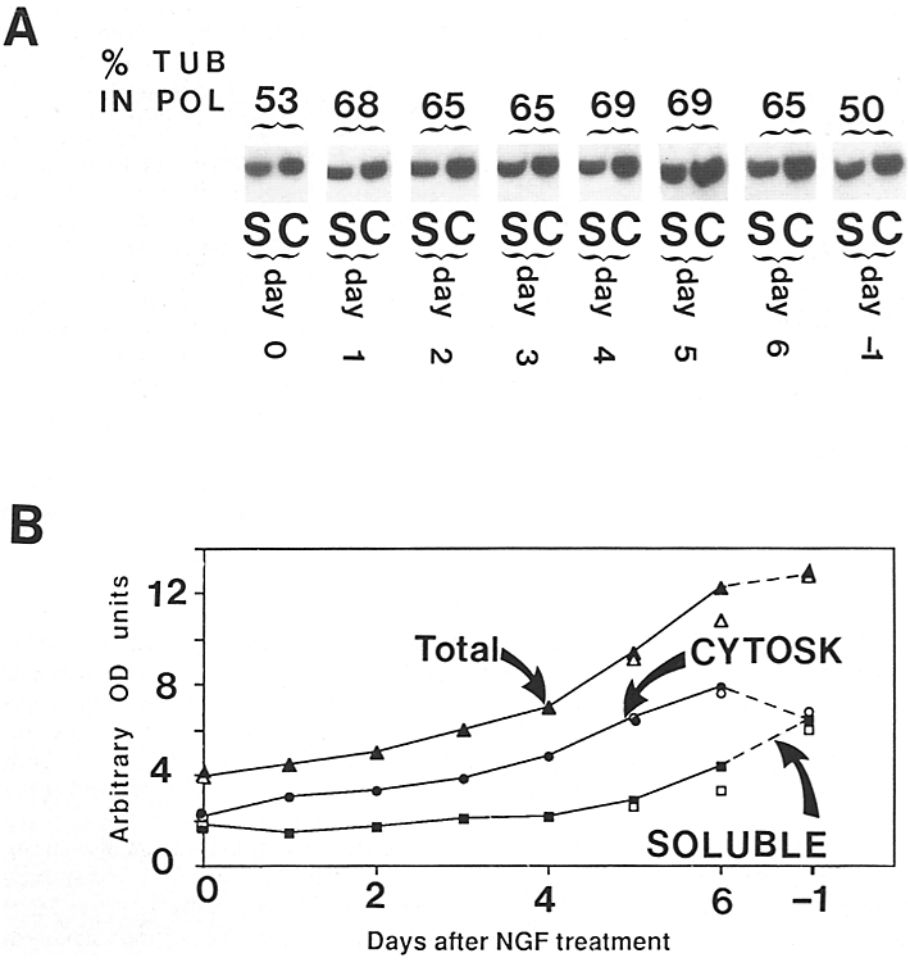


Figure 2. Partitioning of β-tubulins between soluble (*S*) and cytoskeletal (*C*) compartments during NGF-induced neurite outgrowth in PC-12 cells. An equal number of cells (3×10^4 cells/100-mm dish) were plated in eight 100-mm tissue culture dishes. The following day NGF was added to the culture medium (final concentration 100 ng/ml). Extracts containing soluble (*S*) or cytoskeletal (*C*) tubulin were prepared from one 100-mm culture dish of cells on day *0*, *1*, *2*, *3*, *4*, *5*, and *6*, and day *−1* (1 d of NGF withdrawal subsequent to 6 d of NGF treatment). The extracts were brought to equal volumes and protein concentrations were measured in the soluble extracts. 1/10 of each fraction was loaded onto a series of SDS–polyacrylamide gels along with appropriate dilutions of trpE/tubulin fusion proteins. Resultant gels were immunoblotted with a monoclonal antibody that recognizes all the β-tubulin isotypes (*A*). Autoradiograms were scanned by densitometry. (The integrated optical density values were then corrected for protein concentration in the soluble fraction and plotted in *B*.) (■) Soluble β-tubulin; (●) cytoskeletal β-tubulin; and (▲) total β-tubulin levels. (□, ○, △) α-Tubulin polypeptides in the same extracts.

servations). The results from such an experiment are shown in Fig. 2 (solid symbols are for β-tubulin). The proportion of total β-tubulin that is assembled increases rapidly after addition of NGF (from ~50% polymer on day 0 of differentiation to 65% polymer on day 6 of differentiation). A similar situation was found using a monoclonal antibody that probably recognizes all α-tubulins (open symbols in Fig. 2 *B*).

The increased state of polymerization is characteristic of differentiated cells inasmuch as dedifferentiation caused by withdrawal of NGF for 1 d brings the polymer levels back to 50%. We detect no evidence of an increase in unassembled tubulin before neurite outgrowth as had been reported earlier (Drubin et al., 1985).

Quantitative Distribution of β-Tubulin Isoforms in Unassembled and Polymeric Compartments of PC-12 Cells

The increase in the proportion of β-tubulin that is assembled during neurite extension could either be the result of changes in microtubule-assembly properties during neurite extension, could reflect greater assembly of a specific isotype(s), or both. To examine whether one or more of the β-tubulin isotypes accumulated in PC-12 cells are preferentially used for microtubule assembly during neurite outgrowth, the distribution of individual isotypes between the cytoskeletal (microtubule) form and the soluble (unassembled) form was determined using quantitative immunoblotting. PC-12 cells treated with NGF for 5 or 6 d were lysed under conditions that stabilized cellular microtubules to separate soluble proteins from the assembled cytoskeleton. Identical proportions of each fraction were electrophoresed on five parallel gels, along with a series of dilutions of the appropriate cloned fusion proteins corresponding to each β-tubulin isotype. The gels were immunoblotted and the resultant autoradiographs scanned by densitometry.

Data from five independent experiments are summarized in Table I. Although the precise fraction of subunits in polymer varied in the independent experiments (probably the result of small changes in culturing conditions), each experiment showed an identical trend: the cytoskeletal-to-soluble protein ratios (C/S) for isotypes I and II were always significantly higher than for isotypes III and V. Further, in four out of five independent experiments, we observed a C/S ratio for isotype IV comparable to that of isotypes I and II. The cumulative results of these experiments clearly show that for assembly of microtubules in PC-12 cells there is preferential quantitative usage of isotypes I and II (and probably type IV as well). (Although the question of whether all microtubules were in fact stabilized during extraction is problematic, the differences in apparent assembly levels among isotypes is not seriously affected by such concerns since C/S ratios for each isotype have been measured in the same extracts.)

To insure further that these differences did not result from an artifact of nonlinearity in the immunoblot quantitation, we used an alternative procedure that did not rely on fusion protein standards to determine the useful range of the immunoblot assay. A series of 1.25-fold dilutions of soluble and cytoskeletal extracts from a dish of differentiated PC-12 cells were analyzed in parallel by immunoblotting with anti-class I, -class II, -class III, and -class IV antibodies (anti-class V was not analyzed in this experiment). The immunoblots were exposed for 2 d (Fig. 3, *I–IV*). The immunoblot shown in panel II was then reprobed with a monoclonal anti-β-tubulin antibody that recognizes all β-tubulins and exposed for 15 min (shown in Fig. 3, *T*). Densitometry confirmed both the linearity of the assay and the altered degree of assembly of types I, II, and IV as compared with type III. For example, inspection of the figure reveals that soluble dilution *1* and cytoskeletal dilution *5* of isotype II are comparable (i.e., the C/S ratio = 1.25^4:1 = 2.4:1, indicating that ~70% of isotype II is assembled), whereas soluble dilution 1 of isotype III is quantitatively the same as cytoskeletal dilution 2 (i.e., only ~55% is assembled).

Subcellular Localization of Individual β-Tubulin Isotypes in PC-12 Cells

From the quantitative analyses it is clear that tubulin isotypes I, II, and III selectively accumulate, but are differentially utilized for the formation of cellular microtubules as PC-12 cells differentiate from a round soma to an asymmetric morphology. To test whether these induced isotypes and the constitutively expressed isotypes (IV and V) are localized differentially in the soma and the neurite, we stained the microtubule arrays in differentiated PC-12 cells with each of five isotype-specific polyclonal antibodies. Results from this experiment are shown in Fig. 4. The microtubule arrays identified by antibodies to types I–IV (Fig. 4) are qualitatively indistinguishable both in the soma and the neuritic processes, a pattern also seen using a monoclonal antibody that recognizes all β-tubulins (not shown). However, a different pattern was consistently seen for isotype V. Although

*Table I. Quantitative Analysis of Five β-Tubulin Isotypes in the Cytoskeletal Fraction**

Isotype in cytoskeleton	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Experiment 5
	%	%	%	%	%
Class I	69 ± 1 (3)	67 ± 4 (2)	54 (1)	72 (1)	69 ± 1 (3)
Class II	74 ± 1 (3)	68 ± 2 (3)	56 (1)	81 (1)	75 ± 4 (2)
Class III	56 ± 1 (6)	44 ± 3 (6)	35 ± 3 (3)	58 ± 2 (5)	50 ± 1 (3)
Class IV	66 ± 2 (5)	86 ± 4 (4)	52 ± 2 (3)	84 ± 2 (3)	55 ± 1 (2)
Class V	ND	38 ± 1 (3)	38 (1)	60 (1)	54 ± 3 (2)

* Data presented are the mean ± SD. The number of independent determinations is shown in parentheses. Experiments *1–4* represent cells induced with NGF for 6 d; experiment *5* is from cells induced for 5 d.

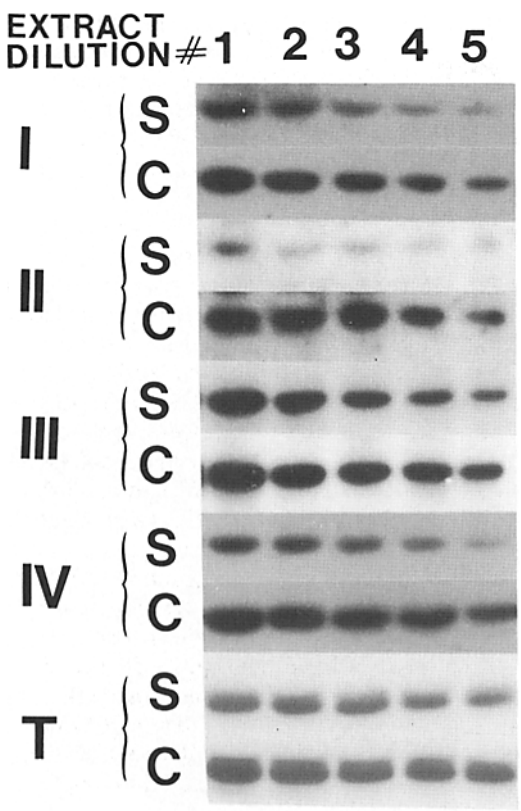


Figure 3. Quantitative analysis of soluble and polymeric β-tubulin isotypes in microtubules of differentiated PC-12 cells by an alternative procedure that does not rely on fusion protein standards. 6 d after NGF treatment, PC-12 cells were fractionated into soluble (*S*) and cytoskeletal (*C*) fractions by lysis under conditions that stabilize microtubules. 1.25-fold serial dilutions (lanes *1–5*) of starting extracts were electrophoresed in four identical gels. Blots were probed with type *I–IV* primary antibodies followed by ^{125}I-protein A. 2-d exposures are shown in *I–IV*. The blot shown in *II* was reprobed with a monoclonal primary antibody that recognizes all the β-tubulin isotypes followed by a "sandwich antibody," a rabbit anti-mouse IgG, and finally by iodinated protein A. This procedure allowed significant amplification of the signal and a 15-min exposure of this immunoblot is shown in *T*.

staining by the type V antibody was evident in both somatic and neuritic microtubules, the relative fluorescence intensity in the soma was clearly higher than that in the processes (Fig. 4, *V*). This partial exclusion of type V from the neurite may explain the quantitative underpolymerization of this isotype.

The staining seen with each individual antibody is due to specific binding of the antibody to the corresponding β-tubulin isotype, since preincubation with the appropriate peptide reduced staining to preimmune background levels, whereas staining was unaffected when any of the nonhomologous peptides was used for preincubation (data not shown).

β-Tubulin Isotype Utilization in PC-12 Cells Is Representative of Authentic DRG Neurons

Differentiated PC-12 cells have been demonstrated to be very similar morphologically and functionally to sympathetic neurons of rat (Greene and Tischler, 1982). To ensure that the β-tubulin isotypic repertoire and the subcellular distribution of each isotype in PC-12 cells is representative of authentic neurons, we performed indirect immunofluorescence on primary cultures of rat sensory neurons. To do this, we isolated DRG from newborn rats and after trypsinization to produce a single cell suspension, primary cultures were established. These were gently extracted with a Triton X-100–containing microtubule stabilization buffer and stained with each isotype-specific antibody. Examination of the fluorescence patterns revealed that the β-tubulin repertoire of these DRG cells and the subcellular distribution of individual isotypes was indistinguishable from that of differentiated PC-12 cells. In particular, isotype V was again found preferentially within the soma (e.g., compare staining of type V and type IV in Fig. 4).

To insure that the bright fluorescence seen in conventional fluorescence microscopy was in fact due to the staining of individual microtubules both in the rounded cell body and in the neuritic processes, we used scanning confocal microscopy to optically section through a rounded cell (White et al., 1987). Confocal images of such an optical cross section through a cell body and a branch point of a neuritic process of DRG cells are shown in Fig. 4, *A* and *B*, respectively. Staining of individual filaments with the type IV antibody is readily apparent. We assume that these fibers represent individual microtubules, as has been demonstrated in other systems (Osborn and Weber, 1982). Similar patterns were found using antibodies to other isotypes, with the exception that individual fluorescent fibers stained with the class I, II, and III antibodies yielded a slightly punctate pattern. The punctate appearance was not visible in bundles of microtubules, but was clearly seen along isolated fibers. An example of such a staining pattern (with the class III antibody) in the soma and in a neurite is shown in the inset of Fig. 4, *A* and *B*, respectively.

Quantitative Immunofluorescence Confirms Partial Exclusion of Isotype V from the Neurite

To confirm the apparent partial exclusion of isotype V from the neurites with a more quantitative assay, we performed the following quantitative immunofluorescence experiment: lysed and fixed cells were successively labeled with type V polyclonal antibody, a monoclonal antibody that recognizes all β-tubulin isotypes, a rhodamine-conjugated anti-rabbit IgG, and a fluorescein-conjugated anti-mouse IgG. Images in both rhodamine and fluorescein channels were directly digitized using a charge coupled device, and analyzed for the ratio of fluorescein to rhodamine intensities pixel-by-pixel with an on-line computer. An example of this quantitative immunofluorescence analysis is shown in Fig. 5 *A*. Comparing the total tubulin signal (in the fluorescein channel) with the amount of type V tubulin (in the rhodamine channel), examination of three independent cells each revealed a fluorescein/rhodamine ratio of >2 in the neurites, in contrast to a ratio of only ~1 in the cell bodies. Clearly, this quantitative analysis confirms the partial exclusion of type V from neurites. As a control, we performed a similar experiment using the type IV polyclonal antibody and the general β-tubulin monoclonal antibody. Results from this experiment (Fig. 5 *B*) showed a uniform fluorescein/rhodamine ratio of ~1 in both the soma and the neurites, thereby confirming a homogenous distribution of type IV tubulin between the soma and neurites.

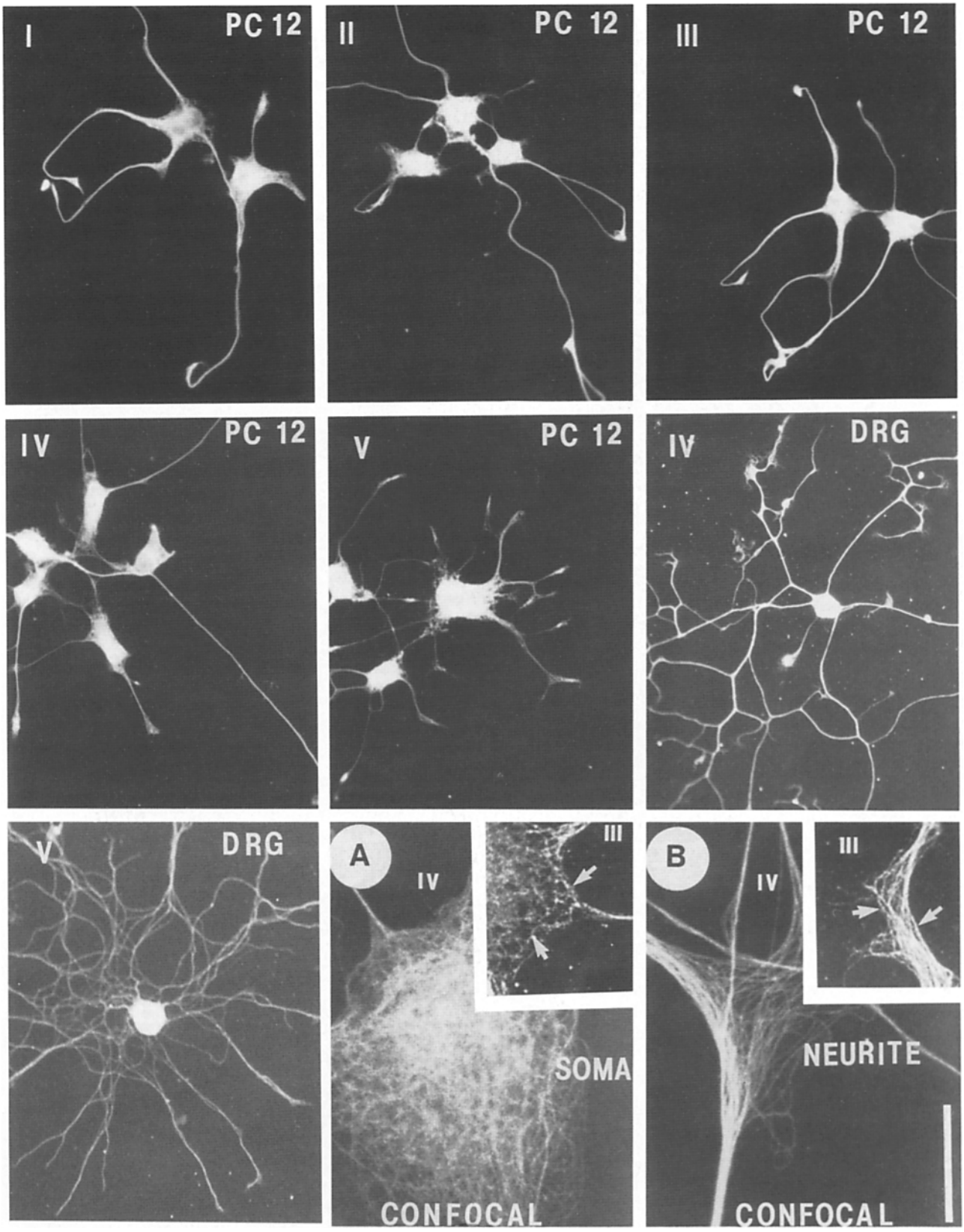


Figure 4. Indirect immunofluorescence visualization of individual β-tubulin isotypes in the microtubules of differentiated PC-12 cells and in primary cultures of DRG neurons using isotype-specific antibodies. (*I–V*) Antibody specific to isotypes I–V, respectively. *A* and *B* are 0.7-μm-thick optical sections of DRG images in the soma (*A*) or neurites (*B*) from confocal, fluorescence microscopy stained with antibody to type IV (main portion of the figure) or to type III (*insets*). Bar, 10 μm.

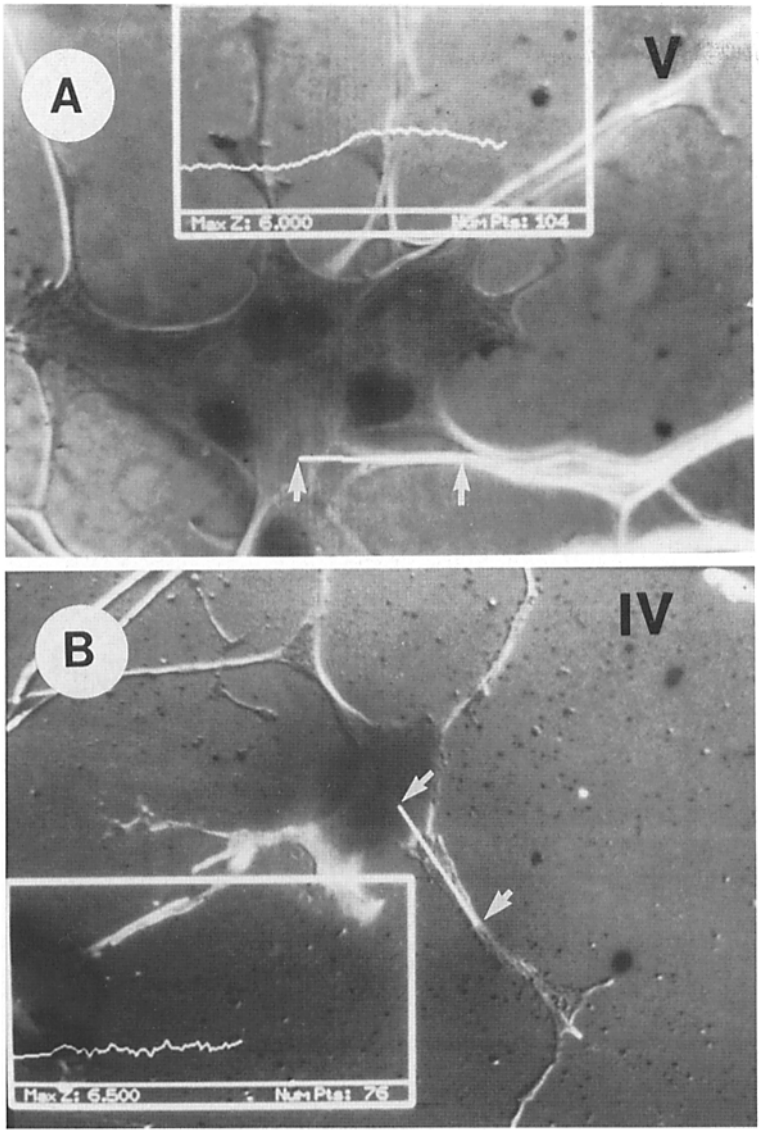


Figure 5. Quantitative immunofluorescence showing homogeneous distribution of class IV tubulin and partial exclusion of class V tubulin from the neurites of PC-12 cells. PC-12 cells were induced by incubation for 5 d in NGF, fixed, and labeled first with rabbit polyclonal antibodies specific to either type V (*A*) or type IV (*B*) β-tubulins. Cells were subsequently reincubated with a mouse monoclonal antibody that recognizes all β-tubulins. The mouse monoclonal antibody was visualized in the fluorescein channel and rabbit antibodies were visualized in the rhodamine channel by the use of appropriate secondary antibodies that were coupled to fluorescein or rhodamine, respectively. Images in both rhodamine and fluorescein channels were digitized by a charge coupled device and further analyzed with an on-line computer. *A* and *B*, *insets*, show a plot of the ratio of fluorescein intensity (total β-tubulin) to rhodamine intensity (class V β-tubulin) as a function of position along a defined line, beginning in the cell body and extending into the neurite (delineated by the arrows).

Immunogold Electron Microscopy Reveals Nonuniform Distribution of Isotype III along Axonal Microtubules

Although some differences in isotype localization were detected by immunofluorescence microscopy (e.g., partial exclusion of isotype V from neurites and a punctate appearance of isotype III), other differences in localization of individual isotypes could easily have been obscured by the limited resolution of light microscopy. To investigate this possibility, we initially used immunogold electron microscopy to visualize each isotype in PC-12 cell neurites. Unfortunately, in our initial efforts class V β-tubulin staining could not be observed with the type V antibody (presumably the consequence of low titer). On the other hand, as expected from the immunofluorescence experiments, staining with the class IV antibody revealed a relatively uniform density of staining along all neurite microtubules (Fig. 6, *IV*). In striking contrast, isotype III (which is quantitatively underpolymerized; see above) showed a remarkably different pattern: in neurite cytoskeletons, antibody binding was distributed in distinct clusters (Fig. 6, *III*). The widths and spacing of these clusters were widely variable. That this nonuniform localization of type III was not an artifact of the immunogold/sectioning protocol was investigated by performing double-immunogold staining using the type III antibody and a β-tubulin monoclonal antibody to all isotypes as primary antibodies and appropriate secondary antibodies linked to different sized gold particles. The monoclonal antibody bound along the entire length of each microtubule, whereas the type III antibody was restricted to discrete domains (not shown).

Discussion

Despite the conservation of specific β-tubulin isotypic sequences throughout vertebrate evolution, an extensive series of previous investigations have failed to document in vivo differences among these isotypes. Rather, most biochemical and genetic evidence has supported the hypothesis that β-tubulin isotypes are functionally interchangeable. While failure to document biochemical differences in vitro could reasonably be attributed to failure of in vitro assays to mimic

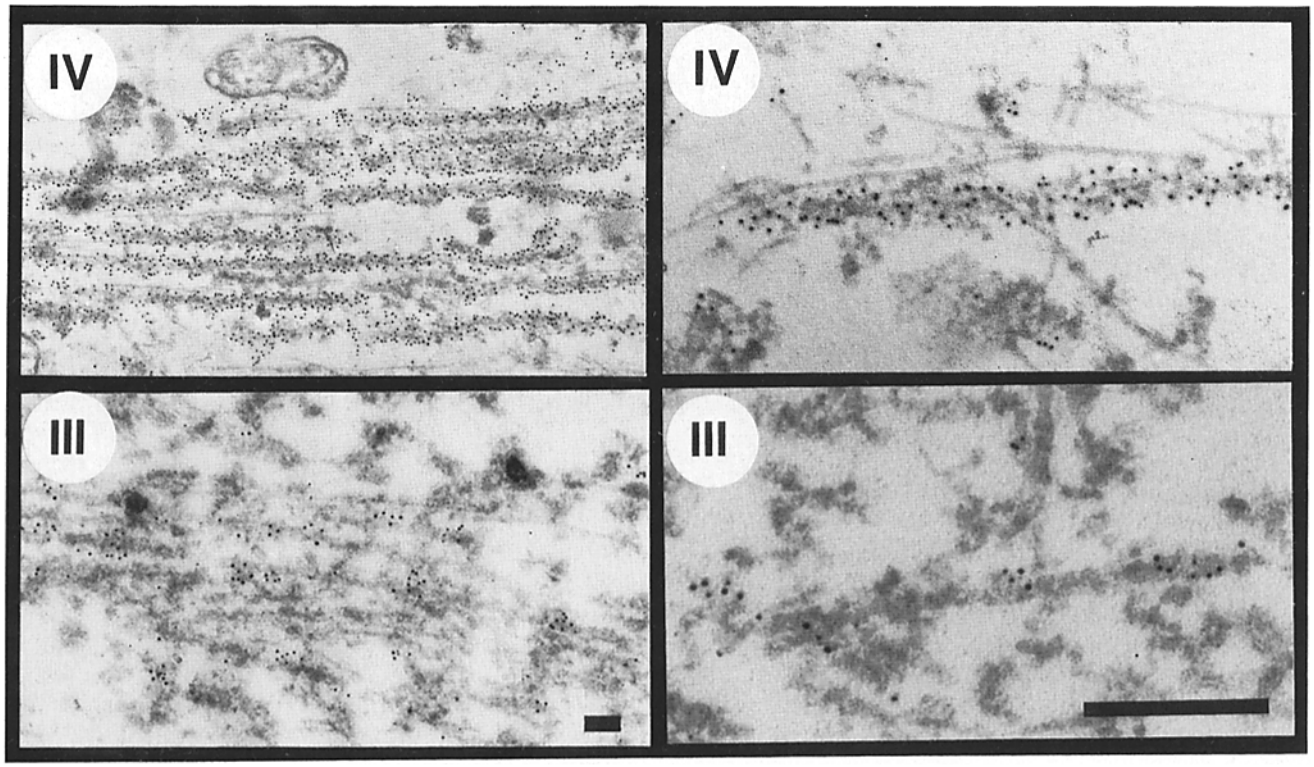


Figure 6. Immunogold localization of β-tubulin isotypes in differentiating neurites. β-Tubulin isotypes IV or III were localized in PC-12 cell neurites using isotype-specific antibodies and secondary antibodies linked to 5-nm colloidal gold. The top panels are isotype IV at low (*left*) or high (*right*) magnification. Lower panels show isotype III localization. Bars, 400 nm.

the in vivo situation accurately, the recent demonstration, using isotype-specific antibodies, that within fibroblastic cells all microtubules appear to be assembled as random copolymers of available isotypes (Lewis et al., 1987; Lopata and Cleveland, 1987; Gu et al., 1988), further suggested the absence of in vivo distinctions. Although it remained possible that subunit distinctions (e.g., binding of specific microtubule-associated proteins) could still be present, the preponderance of the evidence lay in favor of subunit interchangeability.

These previous efforts not withstanding, our present investigation of tubulin isotype utilization during neurite extension in PC-12 cells has uncovered three unambiguous examples of in vivo distinctions among the isotypes.

First, although all five β-tubulin isotypes conserved in vertebrates are used for microtubule assembly both in authentic DRG neurons and in neurites of PC-12 cells, β-tubulin isotypes II and III are preferentially accumulated during neurite outgrowth. While this might have been anticipated since type II is the major neuronal tubulin (Havercroft and Cleveland, 1984; Lewis et al., 1985) and expression of type III is restricted to neurons (Sullivan and Cleveland, 1986; Sullivan et al., 1986), examination of the proportion of each isotype that is polymerized revealed clearly that type II polypeptides are used preferentially for microtubule assembly (along with type I), whereas isotype III is assembled at a lower efficiency (as is type V).

Second, although indirect immunofluorescence localization showed microtubules containing isotypes I–IV to be uniformly distributed between the cell body and the neurites, a twofold higher proportion of assembled isotype V was found in the soma as compared to the neuritic processes. Although we cannot exclude partial masking in the neurite of the epitope recognized by the anti-type V antibody, this finding is consistent with preferential (but incomplete) exclusion of isotype V from the neurites.

Third, unlike class IV tubulin, immunogold staining revealed that the class III isotype was not found uniformly along neurite microtubules, but rather was present in distinct clusters.

The greater use of class I and II isotypes for microtubule assembly (and underassembly of types III and V) may derive from several potential sources. One is that these isotypes may have intrinsic biochemical properties that allow them to polymerize more efficiently under physiological conditions. (Although in vitro evidence for such differences between some isotypes has been documented [Rothwell et al., 1986], increased levels of assembly of type I have not been found in fibroblasts [Lopata and Cleveland, 1987].) Another possibility might be the stabilization of the microtubules enriched in types I and II as a consequence of preferential association with a specific microtubule-associated protein. In particular, we have found tau accumulation to correlate temporally with type I and type II isotypes. This could, in principle, explain earlier findings of Black and Greene (1982) and Heidemann et al. (1985) that microtubule stability is correlated with neurite outgrowth. Whatever the actual mechanisms, microtubules enriched in individual isotypes could be assembled by "kinetic sorting." If particular subunits intrinsically assembled more efficiently or were stabilized in polymer by preferential binding of specific associated proteins, this would produce microtubules that were enriched in one or more iso-

types. (In fact, Kim et al. [1986] have reported a heterogeneous distribution of MAP binding sites on in vitro assembled microtubules.) Potentially, a population of microtubules so enriched might then be used as the preferred substrate for a particular function.

In any event, from consideration of all of the evidence, we conclude that within specialized microtubules of PC-12 cells, β-tubulin isotype composition can specify or reflect some unique in vivo microtubule property.

We thank Margaret Lopata for providing antisera to chicken β-tubulin isotypes I-V and her advice during the course of this study. We also thank Drs. M. DeBrabander and G. Geuens for their hospitality in opening their facilities to us and generously showing us how to do immunoelectron microscopy. We are grateful to Drs. D. Lansing Taylor, M. Nederlos, and K. Ryan (Carnegie Mellon Institute, Pittsburgh, PA) for helping us with quantitative immunofluorescence. We are also grateful to Dr. J. White and Dr. W. B. Amos from the Medical Research Council Laboratory of Molecular Biology (Cambridge, UK) for helping us with confocal imaging analysis in their laboratory. We also thank Dr. M. Kirschner and Dr. D. Dreschel of the University of California at San Francisco and Dr. L. Binder of the University of Alabama for providing antibodies to tau and MAP-2. Finally, we acknowledge Dr. D. Murphy of Johns Hopkins University (Baltimore, MD) for his helpful discussion during the course of the study.

This work has been supported by grants from the National Institutes of Health and American Heart Association to D. W. Cleveland. Dr. H. C. Joshi is supported by a postdoctoral fellowship award from the Maryland Affiliate of the American Heart Association.

Received for publication 25 August 1988 and in revised form 31 March 1989.

Note added in proof: Recently, Arai and Matsumoto (Arai, T., and G. Matsumoto. 1988. *J. Neurochem.* 51:1825–1838) have reported that β-tubulin isotypes are differentially distributed between the peripheral and central axoplasm of squid giant neurons.

References

Black, M. M., and L. A. Greene. 1982. Changes in the colchicine susceptibility of microtubules associated with neurite outgrowth: studies with nerve growth factor-responsive PC12 pheochromocytoma cells. *J. Cell Biol.* 95:379–386.

Bond, J. F., J. L. Fridovich-Keil, L. Pillus, R. C. Mulligan, and F. Soloman. 1986. A chicken-yeast chimeric β-tubulin protein is incorporated into mouse microtubules in vivo. *Cell.* 44:461–468.

Cleveland, D. W. 1987. The multitubulin hypothesis revisited: what we have learned? *J. Cell Biol.* 104:381–383.

Cowan, N. J., S. A. Lewis, S. Sarkar, and W. Gu. 1988. Functional versatility of mammalian β-tubulin isotypes. *In* The Cytoskeleton in Cell Differentiation. R. B. Maccioni and J. Arechaga, editors. IRL Press, Ltd., Oxford, England. 157–166.

Daniels, M. 1975. The role of microtubules in growth and stabilization of nerve fibers. *Ann. NY Acad. Sci.* 253:535–544.

Drubin, D. G., S. C. Feinstein, E. M. Shooter, and M. W. Kirschner. 1985. Nerve growth factor-induced neurite outgrowth in PC-12 cells involves the coordinate induction of microtubule assembly and assembly promoting factors. *J. Cell Biol.* 101:1799–1807.

Drubin, D., S. Kobayashi, D. Kellogg, and M. Kirschner. 1988. Regulation of microtubule protein levels during cellular morphogenesis in nerve growth factor-treated PC12 cells. *J. Cell Biol.* 106:1583–1591.

Edde, B., C. Jeanette, and F. Gros. 1981. One β-tubulin subunit accumulates during neurite outgrowth in mouse neuroblastoma cells. *Biochem. Biophys. Res. Commun.* 3:1035–1043.

Fields, K. L., J. P. Brockes, R. Mirsky, and L. M. B. Wendon. 1978. Cell surface markers for distinguishing different cell types of rat dorsal root ganglion cells in culture. *Cell.* 14:43–51.

Fuller, M. T., J. H. Caulton, J. A. Hutchens, T. C. Kaufman, and E. C. Raff. 1987. Genetic analysis of microtubule structure: a β-tubulin mutation causes the formation of aberrant microtubules in vivo and in vitro. *J. Cell Biol.* 104:385–394.

Fulton, C., and P. A. Simpson. 1976. Selective synthesis and utilization of flagellar tubulin. The multitubulin hypothesis. *In* Cell Motility. R. Goldman, T. Pollard, and J. Rosenbaum, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 987–1005.

Gard, D. L., and M. W. Kirschner. 1985. A polymer-dependent increase in phosphorylation of β-tubulin accompanies differentiation of a mouse neuroblastoma cell line. *J. Cell Biol.* 100:764–774.

Geuens, G., G. G. Gundersen, R. Nuydens, F. Cornelissen, J. C. Bulinski, and M. DeBrabander. 1986. Ultrastructural colocalization of tyrosinated and detyrosinated α-tubulin in interphase and mitotic cells. *J. Cell Biol.* 103:1883–1893.

Greene, L. A., and A. S. Tischler. 1976. Establishment of a noradrenergic clonal cell line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc. Natl. Acad. Sci. USA.* 73:2424–2428.

Greene, L. A., and A. S. Tischler. 1982. PC-12 pheochromocytoma cultures in neurobiological research. *Adv. Cell. Neurobiol.* 3:373–414.

Gu, W., S. A. Lewis, and N. J. Cowan. 1988. Generation of antisera that discriminate among mammalian α-tubulins: introduction of specialized isotypes into cultured cells results in their coassembly without disruption of normal microtubule function. *J. Cell Biol.* 106:2011–2022.

Havercroft, J. C., and D. W. Cleveland. 1984. Programmed expression of β-tubulin genes during development and differentiation in the chicken. *J. Cell Biol.* 99:1927–1935.

Heidemann, S. R., H. C. Joshi, A. Schechter, J. R. Fletcher, and M. Bothwell. 1985. Synergistic effect of cyclic AMP and nerve growth factor on neurite outgrowth and microtubule stability of PC12 cells. *J. Cell Biol.* 100:916–927.

Joshi, H., P. Baas, D. Chu, and S. R. Heidemann. 1986. The cytoskeleton of neurites after microtubule depolymerization. *Exp. Cell Res.* 163:223–245.

Joshi, H., T. Y. Yen, and D. W. Cleveland. 1987. In vivo co-assembly of a divergent β-tubulin subunit (cβ6) into microtubules of different function. *J. Cell Biol.* 105:2179–2190.

Kemphues, K., T. C. Kaufmann, R. A. Raff, and E. C. Raff. 1982. The testis specific β-tubulin subunit in Drosophila melanogaster has multiple functions in spermatogenesis. *Cell.* 31:655–670.

Kim, H., C. G. Jensen, and L. I. Rebhun. 1986. The binding of MAP-2 and tau on brain microtubules in vitro: implications for microtubule structure. *Ann. NY Acad. Sci.* 466:218–239.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.

Landis, S. C. 1983. Neuronal growth cones. *Annu. Rev. Physiol.* 45:567–580.

Lewis, S. A., W. Gu, and N. J. Cowan. 1987. Free intermingling of mammalian β-tubulin isotypes among functionally distinct microtubules. *Cell.* 49:539–548.

Lewis, S. A., M. G. S. Lee, and N. J. Cowan. 1985. Five mouse tubulin isotypes and their regulated expression during development. *J. Cell Biol.* 101:852–861.

Lopata, M. A., and D. W. Cleveland. 1987. In vivo microtubules are copolymers of available β-tubulin isotypes: localization of each of six vertebrate β-tubulin isotypes using polyclonal antibodies elicited by synthetic peptide antigens. *J. Cell Biol.* 105:1707–1720.

Miller, R. H., R. J. Lasek, and M. J. Katz. 1986. Preferred microtubules for vesicle transport in lobster axon. *Science (Wash. DC).* 235:220–222.

Murphy, D. B., K. T. Wallis, P. S. Machlin, H. Ratrie III, and D. W. Cleveland. 1987. The sequence and expression of the divergent β-tubulin in chicken erythrocytes. *J. Biol. Chem.* 262:14305–14312.

Osborn, M., and K. Weber. 1982. Immunofluorescence and immunocytochemical procedures with affinity purified antibodies: tubulin containing structures. *Methods Cell Biol.* 244:98–129.

Raff, E. C. 1984. Genetics of microtubule systems. *J. Cell Biol.* 99:1–10.

Rothwell, S. W., W. A. Grasser, and D. B. Murphy, 1986. Tubulin variants exhibit different assembly properties. *Ann. NY Acad. Sci.* 466:103–110.

Savage, C., M. Hamelin, J. Cuolotti, A. Coulson, D. G. Albertson, and M. Chalfie. 1989. Mec-7 is a β-tubulin gene required for production of 15 protofilament microtubules in C. elegans. *Genes & Dev.* In press.

Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk. 1985. Measurement of protein using bicinchonic acid. *Anal. Biochem.* 150:76–85.

Solomon, F., M. Magendantz, and A. Salzman. 1979. Identification with cellular microtubules of one of the co-assembling microtubule-associated proteins. *Cell.* 18:431–435.

Stephens, R. 1975. Structural chemistry of the axoneme: evidence for chemically and functionally unique tubulin dimers in outer fibers. *In* Molecules and Cell Movement. S. Inoue and R. E. Stephens, editors. Raven Press, New York. 181.

Sullivan, K. F., and D. W. Cleveland. 1986. Identification of conserved isotype-defining variable region sequences for four vertebrate β-tubulin polypeptide classes. *Proc. Natl. Acad. Sci. USA.* 83:4327–4331.

Sullivan, K. F., J. C. Havercroft, P. S. Machlin, and D. W. Cleveland. 1986. Sequence and expression of the chicken β5 and β4 tubulin genes define a pair of divergent β tubulins with complementary patterns of expression. *Mol. Cell. Biol.* 6:4409–4418.

White, J. G., W. B. Amos, and M. Fordham. 1987. An evaluation of confocal versus conventional imaging of biological structures by fluorescence light microscopy. *J. Cell Biol.* 105:41–48.

Yamada, K. M., B. S. Spooner, and N. K. Wessels. 1970. Axon growth: roles of microfilaments and microtubules. *Proc. Natl. Acad. Sci. USA.* 66:1206–1212.