A Polymer-dependent Increase in Phosphorylation of β-Tubulin Accompanies Differentiation of a Mouse Neuroblastoma Cell Line

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ABSTRACT We have examined the phosphorylation of cellular microtubule proteins during differentiation and neurite outgrowth in N115 mouse neuroblastoma cells. N115 differentiation, induced by serum withdrawal, is accompanied by a fourfold increase in phosphorylation of a 54,000-mol-wt protein identified as a specific isoform of β-tubulin by SDS PAGE, two-dimensional isoelectric focusing/SDS PAGE, and immunoprecipitation with a specific monoclonal antiserum. Isoelectric focusing/SDS PAGE of [^{35}S]methionine-labeled cell extracts revealed that the phosphorylated isoform of β-tubulin, termed β_2, is one of three isoforms detected in differentiated N115 cells, and is diminished in amounts in the undifferentiated cells.

Taxol, a drug which promotes microtubule assembly, stimulates phosphorylation of β-tubulin in both differentiated and undifferentiated N115 cells. In contrast, treatment of differentiated cells with either colcemid or nocodazole causes a rapid decrease in β-tubulin phosphorylation. Thus, the phosphorylation of β-tubulin in N115 cells is coupled to the levels of cellular microtubules. The observed increase in β-tubulin phosphorylation during differentiation then reflects developmental regulation of microtubule assembly during neurite outgrowth, rather than developmental regulation of a tubulin kinase activity.

The dynamic nature of microtubules and the multitude of roles they play within the cell make the regulation of the assembly and function of microtubules a central issue in cell biology (for review see reference 1). The existence of multiple isoelectric forms of the α- and β-tubulin subunit proteins has led to the suggestion that subunit heterogeneity may be in part responsible for the regulation of microtubule assembly and function in vivo. For example, in vertebrate brain as many as 17 α- and β-tubulin isoforms have been observed by two-dimensional gel electrophoresis (2, 3). Though this heterogeneity may be partially due to the presence of heterogeneous cell types in brain, as many as six tubulin isoforms have been observed in a clonally derived line of neuroblastoma cells (4) and in single neuronal cells in culture (5).

Recent analysis indicates that some of the observed heterogeneity in tubulin may result from the expression of distinct tubulin genes (6, 7). However, in addition to the genetically encoded differences in α- and β-tubulin polypeptides, there is also ample evidence for the generation of tubulin heterogeneity by posttranslational modifications (8–12). One such modification shared by tubulin and many other cytoskeletal proteins is phosphorylation (9, 10). Protein phosphorylation and phosphorylation cascades have been found to play major roles in the control of many metabolic processes (13, 14). It has been postulated that the observed phosphorylation of structural proteins such as actin (15), intermediate filament proteins (16, 17), myosin (18), tubulin (9, 10), and microtubule-associated proteins (MAPs)[1] (19, 20), might function as a mechanism for regulation of cytoskeletal assembly and

[1] *Abbreviations used in this paper:* DME, Dulbecco's modified Eagle's medium; IEF, isoelectric focusing; IPB, immunoprecipitation buffer (25 mM Tris-Cl [pH 7.5], 2 mM EGTA, 150 mM NaCl, 5 mM NaF, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS); MAP, microtubule-associated protein; MTP, microtubule protein; PB, polymerizing buffer (100 mM 2[*N*-morpholino]ethanesulfonic acid [pH 6.55], 0.5 mM $MgCl_2$, 2 mM EGTA, 0.1 mM EDTA, 1 mM 2-mercaptoethanol, and 1 mM guanosine triphosphate); TCA, trichloroacetic acid.

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function. However, little is currently known regarding the link between tubulin phosphorylation and microtubule assembly and function in vivo.

In this study, we found evidence that a striking phosphorylation of a specific β-tubulin isoform accompanies differentiation and neurite outgrowth in N115 mouse neuroblastoma cells. Furthermore, drugs which affect microtubule assembly were found to alter the observed levels of β-tubulin phosphorylation. Phosphorylation is drastically reduced by colcemid or nocodazole, drugs which disassemble cellular microtubules, and is substantially increased by treatment with taxol, a drug which induces microtubule assembly (21). These data suggest that the level of β-tubulin phosphorylation directly reflects the cellular content of microtubule polymer. We therefore conclude that the increase in tubulin phosphorylation observed during N115 cell differentiation reflects the increased assembly of microtubules during neurite outgrowth.

MATERIALS AND METHODS

Cell Culture: N115 mouse neuroblastoma cells were grown in Dulbecco's modified Eagle's medium (DME) supplemented with 10% calf serum and penicillin-streptomycin (100 U/ml). Differentiation was induced by transfer to DME without serum for the times indicated.

Taxol was obtained from the National Cancer Institute. Stock solutions of 10 mM taxol were prepared in dimethyl sulfoxide and added to DME to final concentrations of 10 μM. Colcemid was obtained from Gibco Laboratories, Grand Island, NY, and used at 1.2 μM. Nocodazole was obtained from Aldrich Chemical Co., Inc., Milwaukee, WI, and used at 10 μg/ml.

Radiolabeling of Cultured Cells: 100-mm culture dishes of cells were rinsed once with DME minus phosphate and methionine. For $^{32}PO_4$-labeling experiments, we incubated cells in 4 ml of DME containing one-tenth the normal phosphate concentration, to which we added 100–200 μCi/ml $^{32}PO_4$ (HCl and carrier-free) (Amersham Corp., Arlington Heights, IL), depending on the experiment. [^{35}S]Methionine labeling was for 20 h in 4 ml of DME containing one-tenth normal methionine and 100 μCi/ml [^{35}S]methionine (900–1,200 Ci/mMol) (Amersham Corp.).

Preparation of Labeled Cell Extracts: Individual dishes of radiolabeled cells were rinsed once with 5 ml of cold phosphate-buffered saline (PBS) (0.13 M NaCl, 2 mM KCl, 8 mM Na_2HPO_4, 2 mM KH_2PO_4 [pH 7.2]) containing 1 μg/ml pepstatin A, 1 μg/ml o-phenanthroline, 0.1 mM phenylmethylsulfonyl fluoride, and 0.1 mM benzamidine-HCl to inhibit proteolysis. Cells were then gently scraped from the culture dish with a rubber policeman into 3 ml of cold PBS and centrifuged for 2 min at 1,000 *g*. The resulting cell pellet was resuspended in 200 μl of cold microtubule polymerizing buffer (PB) (100 mM 2(*N*-morpholino)ethanesulfonic acid [pH 6.55], 0.5 mM $MgCl_2$, 2 mM EGTA, 0.1 mM EDTA, 1 mM 2-mercaptoethanol, and 1 mM guanosine triphosphate) supplemented with protease inhibitors (10 μg/ml of pepstatin A, phenanthroline, leupeptin, and aprotinin; 1 mM phenylmethylsulfonyl fluoride and benzamidine-HCl), 10 mM NaF to inhibit phosphatase activity, and 10 mM ATP. Cells were homogenized with five strokes of a motor-driven teflon-glass homogenizer at ~5,000 rpm. Cell homogenates were centrifuged for 1 h in 5 × 41 mm ultraclear centrifuge tubes (Beckman Instruments, Inc., Palo Alto, CA) in a 50Ti rotor at 50,000 rpm (5°C). Aliquots of the labeled supernatants (cytoplasmic extracts) were prepared for SDS PAGE or isoelectric focusing (IEF)/SDS PAGE, or were enriched for cellular microtubule proteins (MTPs) by co-assembly with brain microtubules (see below).

Trichloroacetic Acid (TCA) Precipitation of $^{32}PO_4$ Protein: Incorporation of $^{32}PO_4$ into total cell protein was quantitated by TCA-precipitation onto Whatman 3MM filter discs (Whatman Laboratory Products Inc., Clifton, NJ). In experiments involving different states of differentiation (such as shown in Figs. 2 and 5), filters were washed twice (10 min each) with 10% TCA at 95°C, a third time with 10% TCA at 25°C, twice with 95% ethanol, and given a final rinse with ether. Dried filters were counted in Econofluor (New England Nuclear, Boston, MA).

For experiments involving a single state of differentiation, we washed filters three times in 10% TCA at 25°C, and rinsed them as above. The ratio of TCA (95°C)-precipitable counts to TCA (25°C)-precipitable counts was found to increase during the first few days of differentiation, due to decreases in RNA synthesis during differentiation. This ratio plateaued a level of ~0.3 after 7 d of differentiation.

Bovine Brain MTP: Bovine brain MTP was prepared by two cycles of assembly according to the method of Shelanski (22) as modified by Weingarten (23). Inclusion of protease inhibitors (in particular pepstatin A) was found to greatly increase the recovery of the high molecular weight MAP-1 species. MTP was stored in 10 M glycerol at −20°C, and subjected to a third cycle of assembly before use.

Enrichment of Cellular MTP by Co-assembly with Bovine Brain Microtubules: To prevent in vitro incorporation of $^{32}PO_4$ into protein during the co-assembly process (by the microtubule-associated cAMP-dependent protein kinase), we removed $^{32}PO_4$-labeled nucleotides from the $^{32}PO_4$-labeled cell extracts (prepared as above) by centrifugation through 3-ml columns of Biogel P6 (Bio-Rad Laboratories, Richmond, CA) equilibrated in PB. We carried out all subsequent steps in the presence of 1 mM ATP to further reduce in vitro labeling, 10 mM NaF to inhibit phosphatases, and protease inhibitors (as above). 10–25-μl aliquots of the desalted cell extracts were added to 75 μl of SDS PAGE sample buffer for later analysis. The remaining 175 μl of the extracts were added to 500–800-μg aliquots of thrice-cycled bovine brain MTP in 150–200 μl of PB, and incubated for 30 min at 37°C to allow microtubule assembly. Microtubules were collected by centrifugation (30 min at 40,000 rpm in a 50Ti rotor at 35°C) and resuspended in 150 μl of cold PB. After depolymerization on ice for 30 min, protein aggregates were removed by centrifugation (40,000 rpm for 30 min at 5°C in a 50Ti rotor). The resulting supernatant was then warmed to 37°C for 30 min to induce microtubule assembly, layered over a 400-μl cushion of 50% (wt/vol) sucrose in PB, and centrifuged for 1 h at 50,000 rpm in a 50Ti rotor at 35°C. The resulting pellets containing $^{32}PO_4$-labeled cellular microtubule proteins were then resuspended in PB, or directly in either SDS PAGE or IEF sample buffers.

Immunoprecipitation of Tubulin and MAP-1 from Labeled Cell Extracts: For immunoprecipitation aliquots of labeled cell extracts in SDS PAGE sample buffer containing equal cpm of TCA-precipitable label were brought to 100 μl with immunoprecipitation buffer (IPB) (25 mM Tris-Cl [pH 7.5], 2 mM EGTA, 150 mM NaCl, 5 mM NaF, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) and preadsorbed for 5 min with 50 μl of 10% *Staphylococcus Aureus* (Pansorbin) (Calbiochem-Behring Corp., San Diego, CA). *S. Aureus* was removed by centrifugation and 20 μl of a 1:10 dilution (in IPB) of either DMβ-1 (β-tubulin) (24) or 7-1.1 (MAP-1) (Asai, D. J., W. C. Thompson, H. Schulman, C. F. Dresden, D. L. Purich, and L. Wilson, manuscript in preparation) monoclonal ascites fluid were added. These amounts of antibody were found to give maximal recovery of labeled tubulin or MAP-1. The samples were incubated with antibody for 30 min on ice, followed by addition of 50 μl of 10% *S. Aureus* which had been preincubated in unlabeled cell extract and MTP, and washed in IP buffer plus 250 mM NaCl and 10 mg/ml bovine serum albumin. After a 5-min incubation on ice, samples were centrifuged twice through 0.5-ml cushions of 1 M sucrose in IP buffer, and then washed a final time in 10 mM Tris-HCl (pH 7.5) containing 5 mM EGTA. The *S. Aureus* pellet was resuspended in either SDS PAGE sample buffer or IEF sample buffer, and electrophoresed. Dried gels were autoradiographed, and labeled tubulin or MAP-1 ws quantitated by scintillation counting of excised gel slices in Econofluor (New England Nuclear).

SDS PAGE and IEF/SDS PAGE: SDS PAGE was performed on 6% or 7% polyacrylamide gels (30:0.8 cross-linking) using the discontinuous Tris-glycine buffer described by Laemmli (25). Separation of α- and β-tubulin was enhanced by raising the pH of the resolving gel to 9.2. Samples were mixed with SDS PAGE sample buffer (100 mM Tris-Cl [pH 6.8], 1% SDS, 1% 2-mercaptoethanol and 15% glycerol) heated in a boiling water bath for 3 min. Molecular weight markers used were myosin heavy chain (220,000), β-galactosidase (130,000), transferrin (90,000), bovine serum albumin (68,000), and ovalbumin (43,000).

IEF/SDS PAGE was performed as described by O'Farrell (26). IEF gels contained 2% ampholines (pH 4–6) (Bio-Rad Laboratories, Richmond, CA). 10–20-μl aliquots of cell extract were brought to 100 μl with IEF sample buffer (8 M urea, 2% Nonidet P-40, 1% 2-mercaptoethanol, and protease inhibitors). The second (SDS PAGE) dimension was as described above.

Autoradiography was on Kodak X-omat AR film (Eastman Kodak Co., Rochester, NY). Du Pont Lightning plus intensifying screens (Du Pont Co., Wilmington, DE) were used for $^{32}PO_4$ autoradiography.

$^{32}PO_4$-incorporation into β-tubulin observed in SDS PAGE of microtubule fractions and IEF/SDS PAGE of whole cell extracts and microtubule fractions was quantitated by scanning densitometry on a Zenith Soft Laser scanning densitometer (LKB Instruments, Inc., Gaithersburg, MD).

Phosphoamino Acid Analysis: $^{32}PO_4$ β-tubulin (isolated by immunoprecipitation and SDS PAGE) and $^{32}PO_4$ MAP-1 (isolated by co-assembly and SDS PAGE) were eluted from gel slices with trypsin as previously described (16) and were hydrolyzed in 6 N HCl for 2 h at 100°C. Samples were spotted on cellulose thin layer chromatography plates (Eastman Kodak Co.) and electrophoresed at pH 1.9 (acetic acid:formic acid:H_2O in 1.5:1.3:50) for 1 h at 1,000 V followed by electrophoresis at pH 3.5 (acetic acid:pyridine:H_2O in 3.3:0.38:500) for 45 min at 1,000 V. $^{32}PO_4$-amino acids were detected by

autoradiography. Unlabeled standards were located by staining with 1% ninhydrin.

RESULTS

Serum deprivation of mouse N115 neuroblastoma cells results in a striking morphological differentiation (see Fig. 1). Over a period of several days these normally rounded cells extend long cellular processes termed neurites, which exhibit many properties of neuronal axons, including the assembly of numerous microtubules (27). This cell line thus provides a convenient model system for studying the cellular biochemistry of the regulation of microtubule assembly and function in vivo.

Analysis of $^{32}PO_4$-labeled N115 Proteins

N115 cells were metabolically labeled with $^{32}PO_4$ after periods of differentiation in serum-free medium for up to 2 wk. Cytoplasmic extracts were prepared from homogenates of labeled cells and enriched for $^{32}PO_4$-labeled microtubule proteins by two cycles of assembly in the presence of unlabeled bovine brain microtubules. Aliquots of the total cytoplasmic supernatant fraction and the microtubule-enriched fraction were then analyzed by SDS PAGE and autoradiography to detect the radiolabeled cellular proteins (Fig. 2).

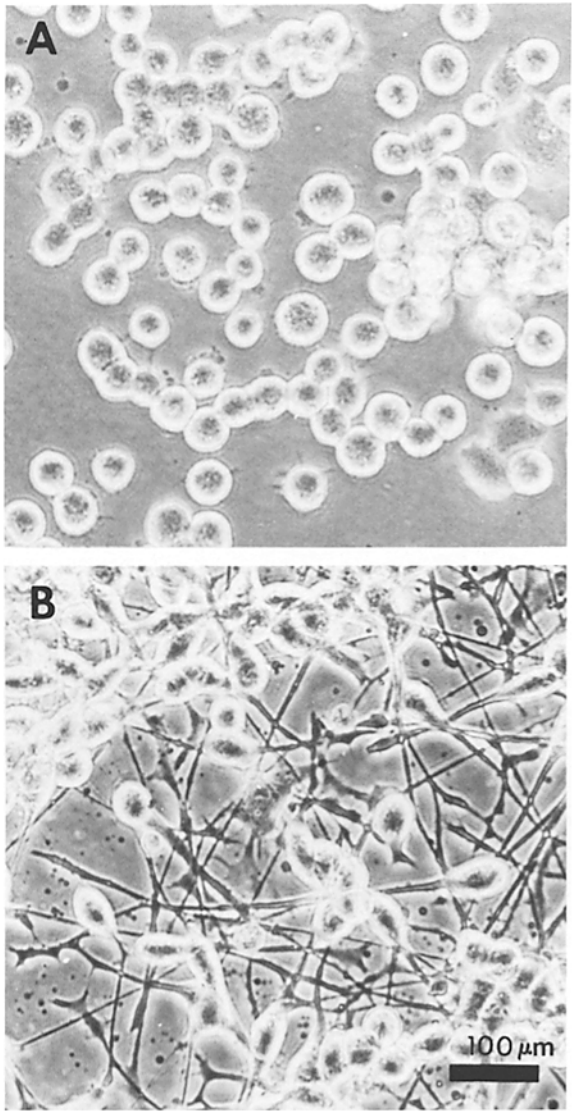


FIGURE 1 Morphological differentiation of N115 neuroblastoma cells in response to serum starvation. Cells grown in the continuous presence of serum (*A*) have a uniform rounded morphology. Serum starvation results in the extension of long neurites, here shown after 7 d (*B*). Bar, 100 μm. × 115.

Initial experiments revealed that in the early stages of N115 differentiation (days 1–4) the incorporation of $^{32}PO_4$ measured by TCA precipitation at room temperature did not accurately reflect the $^{32}PO_4$-incorporation into cell proteins observed by SDS PAGE and autoradiography. This discrepancy is likely due to a decreased contribution of $^{32}PO_4$-labeled RNA to the total incorporation after continued serum starvation. To more accurately quantitate the incorporation on $^{32}PO_4$ into protein over this period of differentiation, we precipitated the $^{32}PO_4$-cell proteins with 10% TCA at 95° (see Materials and Methods) to hydrolyze the labeled RNA, and we normalized gel loads accordingly.

The $^{32}PO_4$-labeled proteins of total cytoplasmic extracts from N115 cells differentiated for 0, 1, 2, 4, 6, 8, 10, and 12 d are shown in Fig. 2*A*. The incorporation of $^{32}PO_4$ into total cell protein appears unchanged through this period of serum starvation. In particular, note the constant level of incorporation into a major phosphoprotein of ~100,000-mol-wt (asterisk). While the overall levels of $^{32}PO_4$-incorporation appear constant, differentiation-specific changes in $^{32}PO_4$-labeling of individual protein species are apparent (small arrows). However, the multitude of $^{32}PO_4$-labeled species prevents any identification of these proteins observed by SDS PAGE.

To visualize possible changes in the phosphorylation of microtubule components during differentiation, we enriched cell extracts for $^{32}PO_4$-microtubule proteins by co-assembly with bovine brain microtubules. SDS PAGE analysis of $^{32}PO_4$-labeled cellular microtubule proteins from N115 cells differentiated for 0, 2, 4, or 8 d is shown in Fig. 2*B*. Several $^{32}PO_4$-labeled proteins have been enriched by the co-assembly process. Those with molecular weights of ~230,000 and 130,000 may represent nonspecific contamination by other cellular components such as neurofilaments, since their presence in the second microtubule pellet was quite variable in different experiments (see, for example, Fig. 4*B*). A 100,000-mol-wt species in the microtubule fraction probably represents contamination by the major cytoplasmic phosphoprotein of the same molecular weight (Fig. 2*A*), and can be used to compare the gel loads in Fig. 2*B*. The 350,000- and 54,000-mol-wt phosphoproteins, on the other hand, were found to efficiently assemble through as many as four cycles of co-assembly with brain microtubules (not shown). The 350,000-mol-wt phosphoprotein co-migrates with the MAP-1 component of brain microtubules (Fig. 2*C*), is heat-labile, and can be immunoprecipitated with a monoclonal antiserum to bovine MAPs (see below), suggesting that it represents the N115 equivalent to the brain MAP-1.

Differentiation-Specific Increase in β-Tubulin Phosphorylation

The 54,000-mol-wt $^{32}PO_4$-labeled protein enriched in the microtubule fraction was found to co-migrate with a prominent component of the bovine brain microtubules (Fig. 2*C*), which is just resolved from brain β-tubulin on SDS PAGE. Incorporation of $^{32}PO_4$ into this species increases substantially during differentiaton. Densitometry reveals that $^{32}PO_4$-incor-

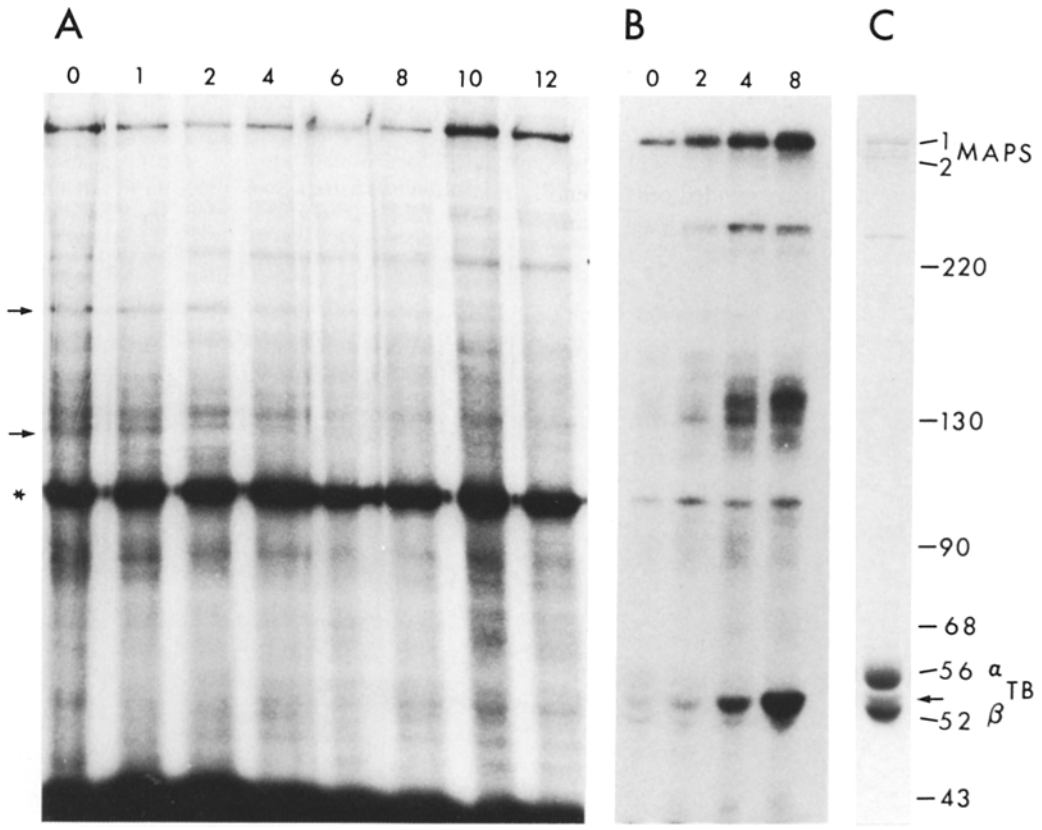


FIGURE 2 (*A*) SDS PAGE analysis of $^{32}PO_4$-labeled cell extracts prepared after 0, 1, 2, 4, 6, 8, 10, or 12 d of serum deprivation (including the 20-h labeling period; 5×10^4 cpm of TCA-precipitable [95°C] $^{32}PO_4$ per lane) reveals little change in the overall pattern of $^{32}PO_4$-incorporation into cellular protein. The major phosphorylated species, with a molecular weight of ~100,000 (asterisk) shows relatively constant labeling throughout differentiation. Changes in labeling of some minor species are apparent (small arrows). (*B*) SDS PAGE of microtubule-enriched fractions from $^{32}PO_4$-labeled cells of 0, 2, 4, and 8 d of differentiation. $^{32}PO_4$-MTPs were enriched by two cycles of assembly with brain microtubules as carrier. Labeled species with molecular weights of 350,000, 230,000, 130,000, and 54,000 are enriched in the microtubule fractions. The 350,000-mol-wt phosphoprotein co-migrates with bovine brain MAP-1. The 54,000-mol-wt species co-migrates with an isoform of bovine brain β-tubulin (arrow in *C*). (*C*) Stained SDS PAGE of the bovine brain MTP used as carrier. The positions of MAPs 1 and 2, α- and β-tubulins, and molecular weight markers are shown. (Autoradiograph in *A* exposed for 4 d with no screen; *B* exposed 2 d with intensifying screen at −70°C.)

poration into the 54,000-mol-wt protein increased fourfold (normalized to the 100,000-mol-wt phosphoprotein) after 8 d of differentiation.

During preliminary characterization of $^{32}PO_4$-microtubule proteins, we noted that the 54,000-mol-wt phosphoprotein co-eluted from phosphocellulose with brain tubulin, and is retained in the tubulin pellet after salt extraction of MAPs from taxol-stabilized microtubules (20) (data not shown). These observations and the numerous accounts of tubulin heterogeneity in neuronal cells suggested that the 54,000-mol-wt cellular phosphoprotein may represent a phosphorylated isoform of tubulin.

To confirm the identity of the 54,000-mol-wt phosphoprotein observed in the microtubule-enriched fraction from N115 cells, we analyzed $^{32}PO_4$-labeled cell extracts and microtubule fractions by two-dimensional IEF/SDS PAGE. Autoradiograms of the IEF/SDS PAGE of total cytoplasmic proteins from labeled undifferentiated cells (Fig. 3*A*) and cells grown without serum for 6 d (Fig. 3*B*) reveal several differences in incorporation of $^{32}PO_4$ into unidentified proteins (small arrows). No incorporation of $^{32}PO_4$ into α-tubulin was observed in extracts (Fig. 3, *A* and *B*) or microtubule fractions (Fig. 3, *C* and *D*) from either undifferentiated or differentiated cells. However, a prominent 54,000-mol-wt phosphorylated species (β_2) migrates near the position of the major β-tubulin species (β_1) in undifferentiated N115 cells. In cells differentiated for 6 d, incorporation of $^{32}PO_4$ into the protein denoted by β_2 is increased 4.6-fold over that observed in undifferentiated cells.

IEF/SDS PAGE of the corresponding fractions enriched for $^{32}PO_4$-labeled cellular microtubule proteins by co-assembly revealed a single 54,000-mol-wt protein species, which is enhanced 4.3-fold in differentiated cells. Comparison of the autoradiograms of the microtubule fractions with the corresponding stained gel (Fig. 3*D* [*inset*]) revealed that the $^{32}PO_4 - \beta_2$ co-migrates with a basic isoform of the bovine brain β-tubulin from the carrier microtubules. The slight separation of this basic isoform in the SDS PAGE dimension corresponds to the splitting of the β-tubulin in the SDS PAGE shown in Fig. 2. From this IEF/SDS PAGE analysis, we concluded that a fourfold increase in $^{32}PO_4$ incorporation into an isoform of β-tubulin accompanies N115 cell differentiation.

Further confirmation of the identity of the $^{32}PO_4 - \beta$-tubulin was provided by immunoprecipitation from $^{32}PO_4$-labeled cell extracts with a monoclonal antiserum specific for β-tubulin. Shown in Fig. 4 are the total $^{32}PO_4$-labeled proteins from a cytoplasmic extract of differentiated N115 cells (Fig.

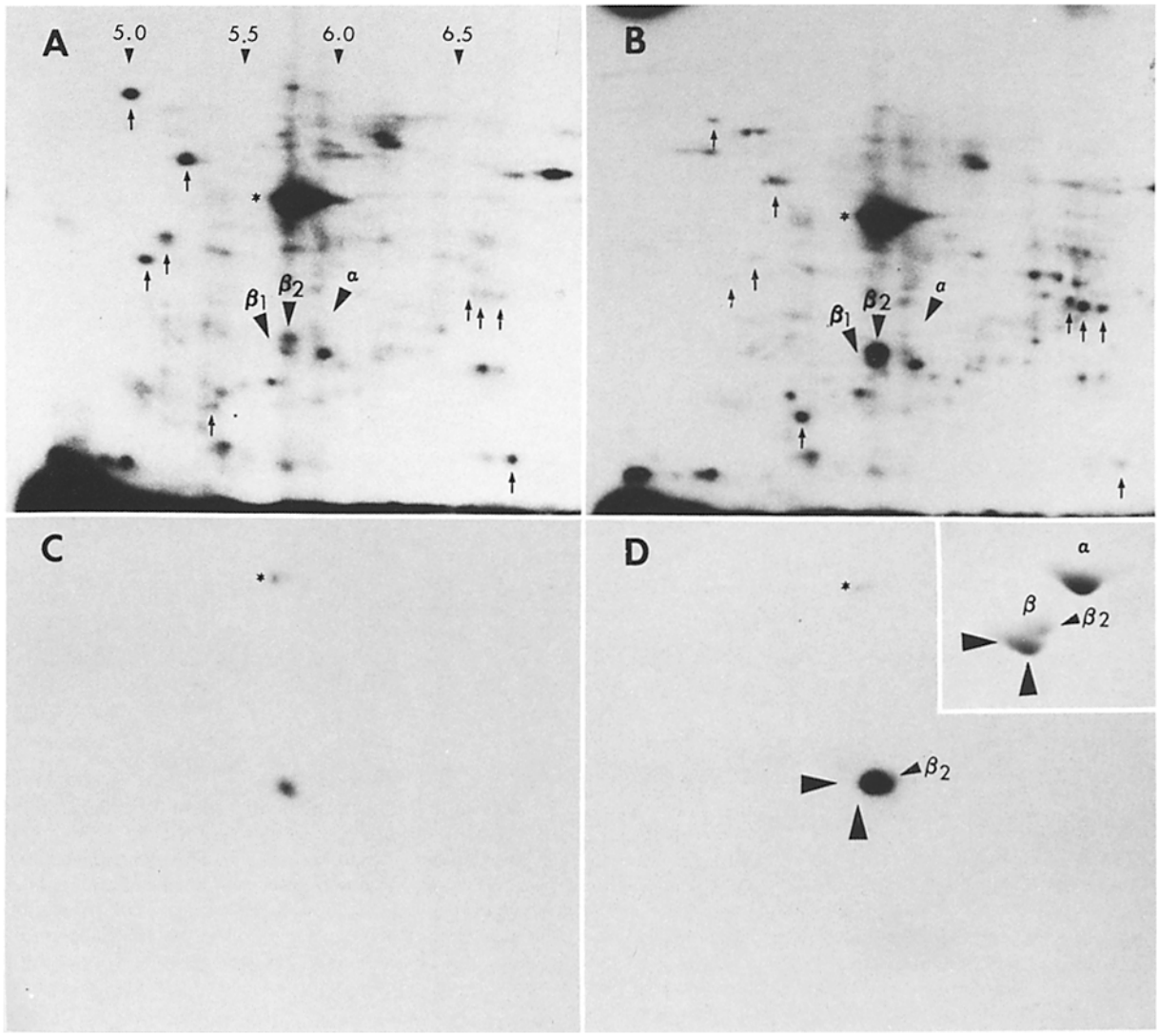


FIGURE 3 (*A* and *B*) IEF/SDS PAGE analysis of $^{32}PO_4$-labeled cell extracts from undifferentiated N115 cells (*A*) and cells grown without serum for 6 d (*B*) reveals numerous differences (both increases and decreases) in phosphate incorporation (small arrows) which were not readily apparent by SDS PAGE alone.The major $^{32}PO_4$-labeled species of 100,000-mol-wt observed in SDS PAGE is readily apparent (asterisk). A 4.6-fold increase in $^{32}PO_4$-labeling of a 54,000-mol-wt species (β_2) migrating near the position of the major N115 β-tubulin (β_1) is observed. No incorporation into α-tubulin (α) is observed. (*C* and *D*) IEF/SDS PAGE of $^{32}PO_4$-labeled microtubule proteins from the above cell extracts are shown in Fig. 3, *C* and *D*. A single $^{32}PO_4$-labeled species with a molecular weight of 54,000 is observed, which shows a 4.4-fold increase in differentiated cells. Comparison of the autoradiogram in *D* with the Coomassie Blue-stained gel (*inset*) reveals that the 54,000-mol-wt protein co-migrates with the most basic of the resolved β-tubulin isoforms (β_2), which also is retarded in the SDS dimension. Slight contamination with the 100,000-mol-wt major phosphoprotein (asterisks) is also apparent.

4*A*): the corresponding microtubule fraction exhibiting the 350,000-mol-wt species and the 54,000-mol-wt β_2-tubulin (Fig. 4*B*), the $^{32}PO_4$-labeled protein precipitated with the monoclonal antiserum DMβ-1, specific for β-tubulin (Fig. 4*C*; provided by Dr. Steve Blose), and for comparison, the $^{32}PO_4$-labeled protein precipitated by the monoclonal 7.1.1 specific for brain MAP-1 (Fig. 4*D*; provided by Dr. David Asai). The 54,000-mol-wt $^{32}PO_4$-β_2-tubulin was specifically precipitated by DMβ-1, and not by 7-1.1, normal rabbit sera, α-tubulin monoclonals, or polyclonal antisera to high molecular weight MAPs (not shown). The precipitation of the 350,000-mol-wt component by the 7-1.1 MAP-1 monoclonal strengthens our identification of this species as an N115 counterpart to brain MAP-1.

We subsequently used immunoprecipitation to quantitate the increase in $^{32}PO_4$-β-tubulin during N115 differentiation. N115 cells were labeled for 20 h with $^{32}PO_4$ in serum-free medium after 0–12 d of prior serum starvation. $^{32}PO_4$-β-tubulin was then immunoprecipitated from aliquots of these extracts containing equal amounts of TCA-precipitable (95°C) (see Materials and Methods) radiolabel with DMβ-1. After SDS PAGE, the immunoprecipitated $^{32}PO_4$-β-tubulin was quantitated by scintillation counting. The results from six independent experiments are presented in Fig. 5. N115 differentiation is accompanied by a fourfold increase in β-tubulin phosphorylation, from 0.1 to 0.4% of the total TCA-precipitable label. The major portion of this increase occurs during the first nine days of differentiation. The magnitude of this increase corresponds well with the four- to fivefold increase estimated from Figs. 2 and 3.

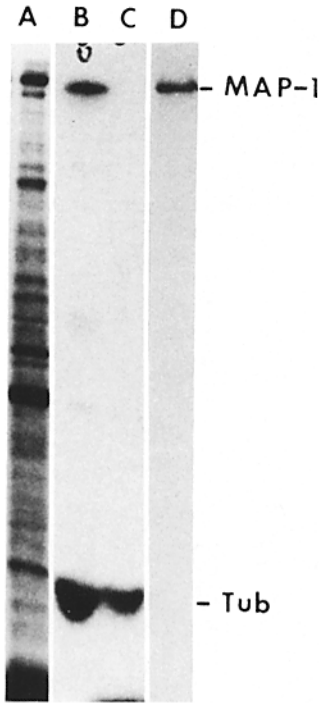


FIGURE 4 Immunoprecipitation of $^{32}PO_4$-tubulin and $^{32}PO_4$-MAP-1 with monoclonal antisera. (*A*) The $^{32}PO_4$-labeled cell extract from differentiated N115 cells (7 d of serum deprivation; 10^4 cpm of TCA-precipitable $^{32}PO_4$). (*B*) The $^{32}PO_4$-microtubule fraction obtained by co-assembly, for identification of the MAP-1 and β-tubulin. (*C*) The DMβ-1 monoclonal anti-β-tubulin specifically immunoprecipitates $^{32}PO_4$-β-tubulin (from 5×10^5 cpm of TCA-precipitable $^{32}PO_4$). (*D*) The monoclonal 7.1.1. anti-MAP-1 specific monoclonal antisera specifically immunoprecipitates a $^{32}PO_4$-labeled species co-migrating with MAP-1.

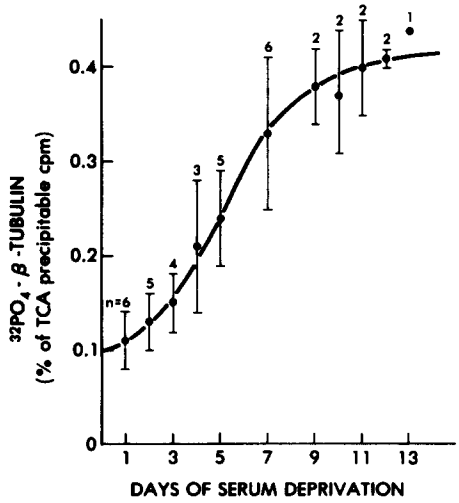


FIGURE 5 Extracts were prepared from differentiating N115 cells labeled for 20 h with $^{32}PO_4$. $^{32}PO_4$-β-tubulin was immunoprecipitaed from aliquots of each extract containing 10^5 cpm of TCA-precipitable (95°C) $^{32}PO_4$. $^{32}PO_4$-β-tubulin was quantitated after SDS PAGE by scintillation counting, and is expressed as the percentage of TCA-precipitable counts. The mean (±SD) and number of determinations for each time point are indicated. The days of differentiation includes the 20-h $^{32}PO_4$-labeling interval.

Analysis of partial amino acid hydrolysates of $^{32}PO_4$-β-tubulin isolated by immunoprecipitation revealed O-phosphoserine as the only phosphorylated amino acid (Fig. 6*A*).

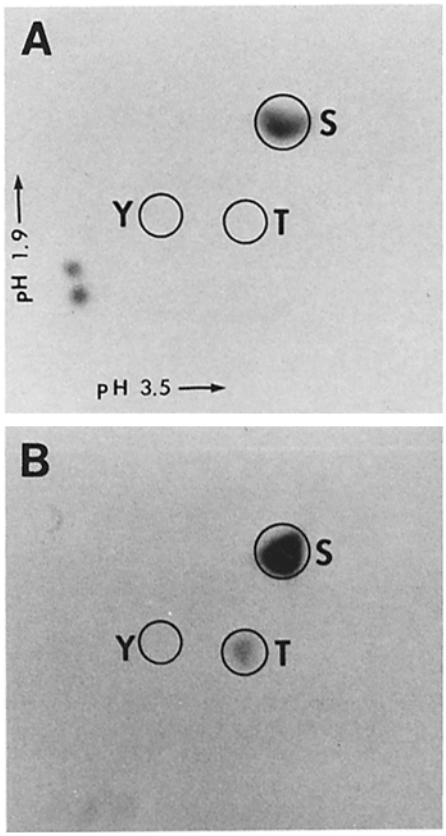


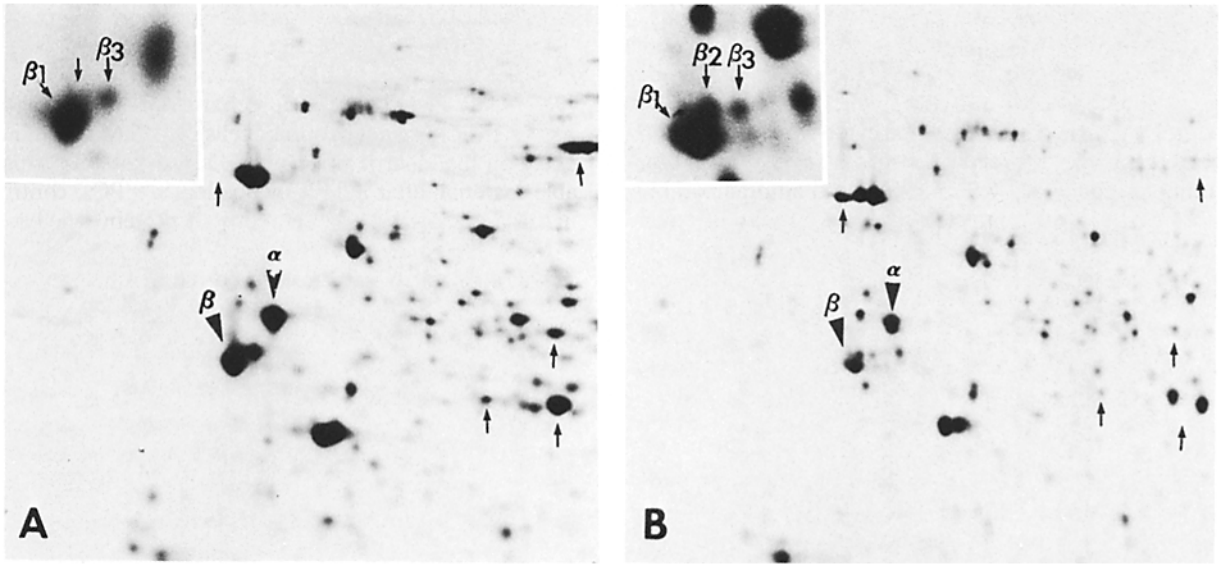
FIGURE 6 Phosphoamino acid analysis of $^{32}PO_4$-β-tubulin and $^{32}PO_4$-MAP-1 from differentiated N115 cells. Two-dimensional high-voltage electrophoresis at pH 1.9 (vertical dimension) and pH 3.5 (horizontal dimension) was used to analyze partial acid hydrolysates of $^{32}PO_4$-β-tubulin (*A*) and $^{32}PO_4$-MAP-1 (*B*). The positions of unlabeled phosphoserine (*S*), phosphothreonine (*T*), and phosphotyrosine (*Y*) are shown. (Autoradiographs exposed 2 d.)

A similar analysis of this N115 MAP-1 species revealed both O-phosphoserine and O-phosphothreonine (Fig. 6*B*).

Appearance of a New β-Tubulin Isoform during N115 Differentiation

To resolve and identify the β-tubulin isoforms present in N115 cells, we metabolically labeled differentiated and undifferentiated cells with [^{35}S]methionine, and prepared cytoplasmic extracts for two-dimensional IEF/SDS PAGE and immunoprecipitation (Fig. 7). IEF/SDS PAGE analysis revealed that, as with $^{32}PO_4$ incorporation, many changes in [^{35}S]methionine-labeling of N115 cell proteins occurred during differentiation (small arrows in Fig. 7, *A* and *B*). When we examined the region of the β-tubulins more closely (see *insets* in Fig. 7, *A* and *B*) we resolved two [^{35}S]methionine-labeled species in undifferentiated cells (β_1 and β_3). Differentiated cells contain an additional species (β_2) which is poorly resolved from the major β-tubulin (β_1). The $^{32}PO_4$-labeled N115 β-tubulin species co-migrates with this β_2-isoform. In addition to the co-migration on IEF/SDS PAGE, the correspondence between the increase in $^{32}PO_4$-β_2-tubulin during differentiation and the increase in ^{35}S-β_2-tubulin further supports our conclusion that these labeled species represent the same polypeptide, which is specific for differentiated N115 cells.

To confirm that β_1–β_3 represent N115 β-tubulins, we im-



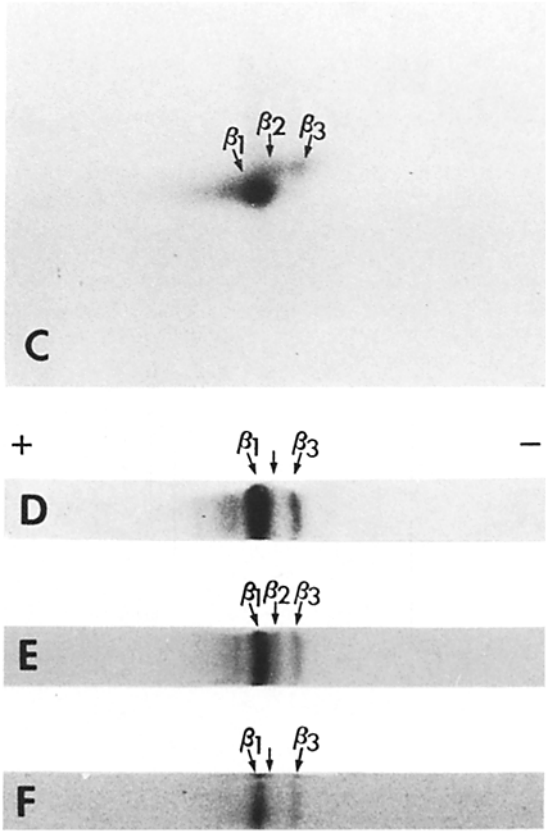


FIGURE 7 (*A* and *B*) IEF/SDS PAGE of extracts from [^{35}S]methionine-labeled undifferentiated N115 cells (*A*), and cells grown without serum for 7 d (*B*). Numerous proteins exhibit differences in [^{35}S]methionine incorporation after 7 d of serum deprivation (small arrows). The position of α- and β-tubulin are indicated. Close examination of the N115 tubulins (*insets*) reveal two putative β-tubulin species (β_1 and β_2) in undifferentiated cells and three species, (β_1, β_2, and β_3) in differentiated cells. The $^{32}PO_4$-β-tubulin co-migrates with the β_2 identified by [^{35}S]methionine labeling. (*C*) To confirm the identification of β_1–β_3, we precipitated the N115 β-tubulins from an extract of [^{35}S]methionine labeled differentiated N115 cells with DMβ-1 monoclonal antiserum. β_1–β_3 were the only species observed. (*D–F*) IEF analysis of [^{35}S]methionine-β-tubulin immunoprecipitated from undifferentiated N115 cells, differentiated N115 cells (8 d without serum), and differentiated cells treated with 1.2 μM colcemid for 4 h at the end of the labeling period. The positions of β_1-, β_2-, and β_3-tubulins are shown in *E*. β_2-Tubulin is not detected in undifferentiated cells (*D*) or in colcemid-treated cells (*F*). Autoradiograms in *A* and *B* exposed for 18 h. (*C–F*) Exposed 5 d. IEF was from right to left in all gels.

munoprecipitated them from cytoplasmic extracts of [^{35}S]-methionine-labeled N115 cells with the DMβ-1 monoclonal antibody and analyzed them by IEF/SDS PAGE. Only three ^{35}S-labeled species were detected in immunoprecipitates from differentiated cells, corresponding to β_1, β_2, and β_3 tubulins. Some loss of resolution was observed after IEF/SDS PAGE of the immunoprecipitated tubulins. Significantly better resolution was obtained by omission of the SDS PAGE dimension, and direct autoradiography of the IEF gels of the immunoprecipitated tubulin (Fig. 4, *D–F*, shown at the same enlargement and orientation as 4*C*). Undifferentiated cells (Fig. 4*D*) contain only β_1, and β_3-tubulin; no β_2 was detectable. Differentiated cells (Fig. 4*E*) contained β_1 and β_3-tubulins, however β_2 was also present in amounts equal to that of β_3, as was observed in the IEF/SDS PAGE of whole cell extracts (Fig. 4*B*). Treatment of ^{35}S-labeled cells with 1.2 μM colcemid, which causes dephosphorylation of β-tubulin (see below) resulted in the loss of immunoprecipitable ^{35}S-β_2-tubulin (Fig. 4*F*).

Taxol Induces an Increase in β-Tubulin Phosphorylation

To address the relationship between phosphorylation and microtubule assembly, we determined the effect of drugs

which alter microtubule assembly on the phosphorylation of tubulin and MAP-1 in N115 cells. For the experiments shown in Fig. 8, we labeled cells for 20 h in $^{32}PO_4$ in the continuous presence of either taxol (10 μM), colcemid (1.2 μM), or nocodazole (10 μg/ml). We prepared cytoplasmic extracts and enriched them for $^{32}PO_4$-MTP by co-assembly with brain microtubules, followed by SDS PAGE and autoradiography. 10 μM taxol, which promotes extensive assembly of microtubules in cultured cells (21, 33), results in a significant increase in $^{32}PO_4$-incorporation into β-tubulin in undifferentiated (compare Fig. 8, lanes *A* and *B*) and differentiated (compare Fig. 8, lanes *C* and *D*) cells compared with parallel cultures labeled in the absence of taxol. Taxol had no apparent affect on the morphology of either differentiated or undifferentiated N115 cells observed by phase-contrast microscopy (not shown).

To further characterize the effect of taxol on β-tubulin phosphorylation, we pretreated differentiated N115 cells for 20 h with taxol (10 μM) after which the time course of $^{32}PO_4$ incorporation (in the continuous presence of 10 μM taxol) into total TCA-precipitable (25°C) material (see Materials and Methods) and into immunoprecipitable β-tubulin or MAP-1 was assessed. Preincubation of cells in 10 μM taxol had little effect on the incorporation of $^{32}PO_4$ into total TCA-precipitable material; after a 24-h incubation in $^{32}PO_4$, control cells had incorporated 2.5×10^5 cpm/μg protein whereas taxol-treated cells incorporated 2.7×10^5 cpm/μg. However, as shown in Fig. 9, taxol increased both the initial rate and final extent of $^{32}PO_4$-incorporation into β-tubulin ~twofold when compared with untreated control cultures. Note, however, that treatment with taxol did not alter the time required to reach either the half-maximal labeling (~4 h) or apparent saturation of labeling (~9 h).

In contrast to the results with β-tubulin, taxol was observed to have no effect on the rate or extent of incorporation of $^{32}PO_4$ into the MAP-1 in N115 cells (Fig. 9).

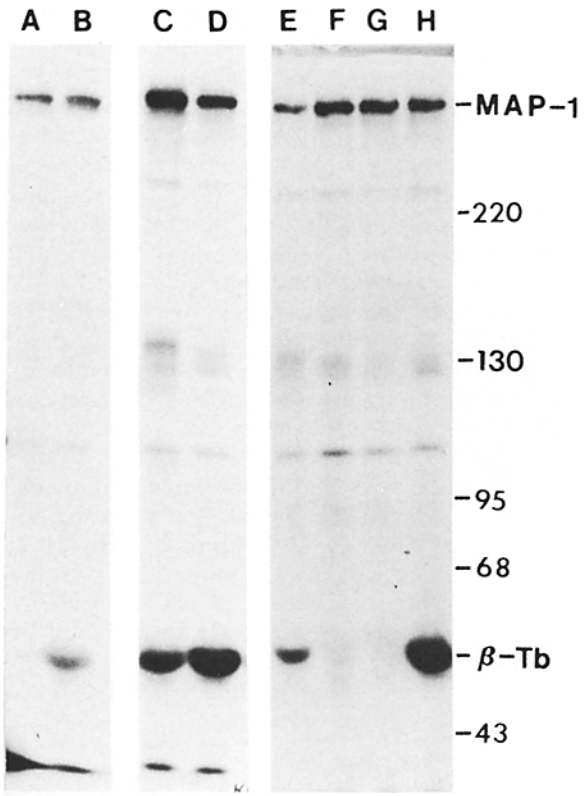


FIGURE 8 SDS PAGE analysis of the microtubule fractions from cells labeled with $^{32}PO_4$ in the presence of 10 μM taxol, 1.2 μM colcemid, or 10 μg/ml nocodazole. Lanes *A* and *B*: Microtubule fraction from undifferentiated N115 cells labeled in the absence (*A*) or presence (*B*) of 10 μM taxol. A dramatic increase in β-tubulin phosphorylation is evident with no apparent change in MAP-1. Lanes *C* and *D*: Microtubules from differentiated N115 cells (7 d without serum) labeled in the absence (*C*) or presence (*D*) of 10 μM taxol. Note the increase in β-tubulin phosphorylation. Lanes *E* and *F*: Microtubules from differentiated N115 cells labeled with $^{32}PO_4$ in the absence of drugs (*E*), or with 1.2 μM colcemid (*F*), 10 μg/ml nocodazole (*G*), or 10 μM taxol (*H*). Colcemid and nocodazole dramatically reduce $^{32}PO_4$-labeling of β-tubulin, with little effect on other species. Taxol induces an increase in β-tubulin phosphorylation, typically two- to threefold (when normalized to MAP-1 phosphorylation). The positions of β-tubulin, MAP-1, and molecular weight markers were determined by Coomassie Blue staining. Autoradiograph exposed 48 h.

Drug-induced Microtubule Disassembly Results In Decreased Phosphorylation of Tubulin

Inclusion of colcemid (Fig. 8*F*) or nocodazole (Fig. 8*G*) during the $^{32}PO_4$-labeling of differentiated N115 cells results in a marked decrease in phosphate incorporation into β-tubulin compared with control cultures (Fig. 8*E*). For comparison, the effect of 10 μm taxol is shown again in Fig. 8*H*. Morphologically, colcemid and nocodazole also result in neurite retraction after relatively short (1–2 h) periods of treatment (26). When differentiated N115 cells are prelabeled for 18–20 h with $^{32}PO_4$ subsequent addition of colcemid (1.2 μM) or nocodazole (10 μg/ml) resulted in a rapid decrease in $^{32}PO_4$-β-tubulin (Fig. 10). $^{32}PO_4$-β-tubulin falls to 50% of the control value within 30 min, decreasing more slowly thereafter, and reaching 20% of the control value after 5–6 h. Examination of β-tubulin immunoprecipitated from extracts of [^{35}S]methionine-labeled cells incubated with (Fig. 6*F*) or

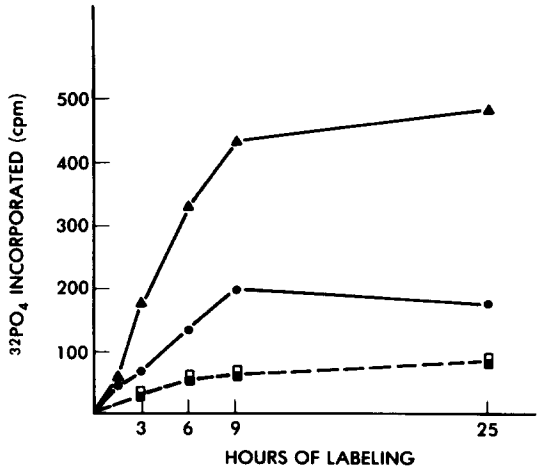


FIGURE 9 Taxol increases $^{32}PO_4$ incorporation into β-tubulin but not MAP-1. Identical cultures of differentiated cells (9 d without serum) were incubated 20 h in the absence or presence of 10 μm taxol, and were subsequently metabolically labeled for the indicated times with $^{32}PO_4$. Incorporation of $^{32}PO_4$ into β-tubulin or MAP-1 was determined by immunoprecipitation from extracts, followed by SDS PAGE and scintillation counting. Results are presented as the number of cpm of $^{32}PO_4$-β-tubulin or MAP-1 immunoprecipitated by the respective antiserum. β-Tubulin: (●) Control; (▲) taxol. MAP-1: (■) Control; (□) taxol.

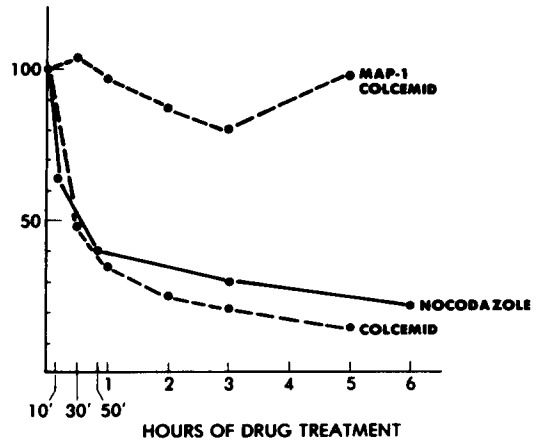


FIGURE 10 Colcemid and nocodazole reduce $^{32}PO_4$-labeling of β-tubulin but not MAP-1. Identical cultures of differentiated N115 cells (7 d without serum) were metabolically labeled for 20 h with $^{32}PO_4$. After subsequent addition of colcemid (1.2 μM) or nocodazole (10 μg/ml), $^{32}PO_4$-labeled β-tubulin and MAP-1 were analyzed at the indicated times by IP (5×10^5 cpm of TCA-precipitable (25°C) $^{32}PO_4$ per sample) followed by SDS PAGE and scintillation counting. Results are presented as the percent labeling compared to the untreated control cultures.

without (Fig. 6 *E*) colcemid revealed that colcemid treatment also resulted in a reduction or loss of the differentiation-specific β_2-tubulin isoform.

Phosphorylation of the N115 MAP-1 protein is unaffected by either colcemid or nocodazole (Fig. 8, lanes *F* and *G*; and Fig. 10).

DISCUSSION

Phosphorylation of a Specific β-Tubulin Isoform Accompanies N115 Differentiation

Differentiation of N115 neuroblastoma cells induced by serum starvation is accompanied by a fourfold increase in $^{32}PO_4$ incorporation into a 54,000-mol-wt cellular phosphoprotein. Several criteria have been used to identify this 54,000-mol-wt cellular phosphoprotein as an isoform of β-tubulin: (*a*) co-assembly with bovine brain microtubules through four cycles of assembly; (*b*) co-migration with a β-tubulin variant found in bovine brain microtubules in both SDS PAGE and IEF/SDS PAGE; (*c*) association with microtubules after high salt extraction of MAPs from taxol-stabilized microtubules; and (*d*) immunoprecipitation by a β-tubulin–specific monoclonal antibody. It is unlikely that the observed increase in $^{32}PO_4$ incorporation into β-tubulin is due to a change in specific activity of the cellular ATP pool during differentiation, since $^{32}PO_4$-incorporation into many other cellular proteins is unaffected. Additionally, the amount of radiolabeled cell extract used in our immunoprecipitations was normalized to circumvent differences in total incorporation of $^{32}PO_4$ into cell protein. It is also unlikely that the reduced amount of $^{32}PO_4$-β-tubulin recovered from undifferentiated cells was due to hydrolysis during sample preparation. Both sodium fluoride (10 mM) and β-glycerophosphate (1 mM) were routinely used to inhibit phosphatase activity during preparation of cell extracts and immunoprecipitations. Preparations of extracts from mixtures of unlabeled-undifferentiated N115 cells with $^{32}PO_4$-labeled differentiated cells had no effect on recovery of $^{32}PO_4$-β-tubulin by immunoprecipitation (D. L. Gard, unpublished observations). Finally, the $^{32}PO_4$-labeling incubations used (18–24 h) were generally more than twice as long as was required for steady-state labeling (9 h) (Fig. 9). Thus we conclude that the increase in $^{32}PO_4$-incorporation which occurs during differentiation reflects an actual increase in the molar levels of phosphorylated β-tubulin.

Two-dimensional IEF/SDS PAGE of N115 $^{32}PO_4$-β-tubulin isolated by co-assembly (Fig. 4) revealed that the phosphorylated species of β-tubulin corresponds to a specific β-tubulin isoform, termed β_2, which is slightly more basic than the predominant isoform of β-tubulin (β_1) in both N115 cells and bovine brain. Since addition of a negatively charged phosphate residue causes an acidic shift in the isoelectric point of a protein, it is unlikely that phosphorylated β_2-tubulin is derived from the more acidic major β-tubulin isoform.

The β_2-tubulin isoform may therefore be derived from a third, more basic form of β-tubulin. A candidate for the unphosphorylate precursor was identified by two-dimensional IEF/SDS PAGE and immunoprecipitation of ^{35}S-labeled β-tubulin from N115 cells. Undifferentiated N115 cells were found to contain two β-tubulins, the major β-tubulin (β_1), and a basic isoform termed β_3. Differentiated N115 cells, however, contain the phosphorylated β_2-tubulin isoform in addition to β_1 and β_3. This phosphorylated β_2-tubulin undoubtedly corresponds to the differentiation-specific isoform of β-tubulin described by Edde et al. (4), which was found to be derived by posttranslational modification of an unknown precursor. Although we have not provided conclusive proof of a precursor/product relationship between β_3 and β_2, β_3 was the only β-tubulin species more basic than β_2 observed by immunoprecipitation with the β-tubulin monoclonal. In addition, the difference in isoelectric point between these two species is consistent with that caused by addition of a single phosphate (28). This suggests that the β_3-tubulin isoform serves as the precursor for the phosphorylated β_2 isoform. Though poorly resolved from the predominate β-tubulin in N115 cells, we estimate that β_2 and β_3 tubulins account for 30% of the total β-tubulin (D. L. Gard, unpublished observations).

Phosphoamino acid analysis reveals O-phosphoserine as the only ^{32}P-labeled species in partial hydrolysates of $^{32}PO_4$-β-tubulin from N115 cells, while preliminary tryptic peptide analysis suggests a single site of phosphorylation (D. L. Gard, unpublished observation).

Phosphorylation of β-Tubulin is Coupled to Microtubule Assembly

Though phosphorylation of α- and β-tubulins have been previously observed (9, 10, 29, 30), little is known of the relationship of these modifications to microtubule assembly or organization. The correspondence between the time of neurite outgrowth during N115 cell differentiation (27) and the oberved increase in β-tubulin phosphorylation suggests a link between β-tubulin phosphorylation and the assembly of microtubules which accompanies neurite outgrowth. The relationship between microtubule polymerization and β-tubulin phosphorylation was determined by assessing the effect of the microtubule-acting drugs colcemid, nocodazole, and taxol on β-tubulin phosphorylation. We found that treatment of cells with colcemid or nocodazole, drugs which cause a rapid depolymerization of cellular microtubules and retraction of neurites in differentiated N115 cells (27), results in a rapid

loss of phosphorylated β-tubulin. The kinetics of the loss of $^{32}PO_4$-β-tubulin induced by colcemid are similar to the kinetics of drug-induced microtubule disassembly in other cultured cell lines (31), and are much faster than phosphate turnover rates in untreated cells (see below), suggesting that the rate of dephosphorylation of tubulin monomer may be limited by the depolymerization process.

On the other hand, treatment of either undifferentiated or differentiated cells with taxol, which promotes extensive microtubule assembly (21, 22), resulted in increased phosphorylation of β-tubulin over control cultures. This taxol-induced increase in β-tubulin phosphorylation is apparent within 1 h of taxol addition to either undifferentiated or differentiated cells (D. L. Gard, unpublished observations). Both the incorporation of $^{32}PO_4$ into β-tubulin and the taxol-induced increase in incorporation are independent of protein synthesis (unpublished observations), suggesting that the observed effects of colcemid and taxol are not a result of changes in tubulin synthesis, such as occurs in some cell lines in response to changes in the tubulin monomer-polymer ratio (31).

Several important conclusions can be derived from these results. First, β-tubulin phosphorylation in N115 cells appears to be closely coupled to the amount of cellular microtubule polymer, as evident in the dramatic effects that colcemid, nocodazole, and taxol have no $^{32}PO_4$ incorporation. Thus the increase in β-tubulin phosphorylation accompanying N115 cell differentiation may reflect increased microtubule polymer levels resulting from assembly of microtubules during neurite outgrowth. This would suggest that significant changes in the tubulin monomer/polymer ratio occur during neuronal differentiation. Such changes are supported by many previous studies in which extracts from differentiated neuroblastoma cells or brain have a greater capacity to support tubulin assembly than extracts from undifferentiated cells (33–36), and by direct measurement of microtubule polymer levels during neuroblastoma differentiation (37).

The ability of taxol to induce increased phosphorylation of β-tubulin in undifferentiated N115 cells further suggests that tubulin phosphorylation is coupled to levels of microtubule polymer during differentiation, rather than reflecting a differentiation-specific increase in a tubulin kinase activity.

Comparison of the time course of $^{32}PO_4$ incorporation into differentiated N115 cells in the presence or absence of taxol (Fig. 9) suggests that β-tubulin-phosphate can turn over on microtubules, without requiring disassembly. If turnover occurred only in monomer, taxol should dramatically slow the incorporation of $^{32}PO_4$ into β-tubulin. In fact, exactly the opposite result was obtained; taxol induces an increase in both the rate and final extent of $^{32}PO_4$ incorporation into β-tubulin. From the incorporation time course (in Fig. 9) we conclude that tubulin-phosphate turns over with a half-life of ~4 h in both control and taxol-treated cells. This lifetime is significantly shorter than the turnover rate of the tubulin polypeptide in other cultured cells (38, 39), suggesting that a given tubulin molecule can go through multiple cycles of phosphorylation-dephosphorylation.

The coupling of phosphorylation of β-tubulin to microtubule polymer levels could occur through several distinct mechanisms. The simplest of these invokes the presence of either a tubulin kinase activity which preferentially recognizes β-tubulin present in the microtubule polymer, or a protein phosphatase which discriminates between monomer and polymer. The rapid loss of $^{32}PO_4$ from β-tubulin during colcemid- or nocodazole-induced microtubule disassembly ($t_{1/2} \simeq 30$ min) compared with the normal turnover ($t_{1/2} \simeq 4$ h) is more easily explained by the latter hypothesis. The level of tubulin phosphorylation could be coupled to cellular microtubule levels through the slower hydrolysis of tubulin-phosphate present in polymer. Increases in cellular microtubule polymer, induced by neurite outgrowth or artificially with taxol, would result in "trapping" of a greater amount of β-tubulin in the phosphorylated form.

We cannot exclude the possibility that a polymer-dependent tubulin kinase is present in these cells. As yet, we have little information regarding the kinase activity responsible for phosphorylating tubulin. While β-tubulin has been shown to serve as a substrate for the $pp60^{src}$ tyrosine kinase in vitro (40), β_2-tubulin is phosphorylated exclusively on serine in vivo. The Ca^{+2}-dependent phosphorylation of tubulin by calmodulin-dependent brain kinases occurs on both α- and β-subunits (29, 30). The cAMP-dependent protein kinase associated with MAP-2 does not significantly phosphorylate tubulin in vitro (19, 20). Knowledge of the actual mechanics of the observed polymer dependent phosphorylation of tubulin awaits the identification of both β-tubulin kinase and phosphatase activities, and an in vitro analysis of their substrate specificities.

The functional role of the observed polymer-dependent phosphorylation of tubulin remains unknown. There is no evidence for an effect of phosphorylation on the ability of β-tubulin to co-assemble with bovine brain microtubules. The changes in tubulin phosphorylation with taxol or colcemid treatment indicate that phosphorylation levels are dependent upon polymer levels, not the reverse, suggesting that phosphorylation is not directly involved in the regulation of microtubule assembly. Though we have not rigorously determined the extent of β-tubulin phosphorylation in vivo, our observations suggest that a significant proportion of the specific β-tubulin polypeptide is actually phosphorylated. The β_2- and β_3-tubulin isoforms are present in approximately equal amounts in differentiated cells. If these isoforms represent the phosphorylated and unphosphorylated forms of the same polypeptide, then ~50% of that polypeptide is phosphorylated. Independent calculations based upon the incorporation of $^{32}PO_4$ also indicated that at least 20% of this polypeptide is phosphorylated in differentiated cells. Since phosphate incorporation is restricted primarily to β-tubulin in polymer, as much as 40–100% of this β-tubulin polypeptide present in polymer could be phosphorylated (based on estimates obtained from other cell lines that 50% of cellular tubulin is in the polymer form [37, 39, 41]). This is also consistent with our observation that treatment with taxol, which should drive virtually all cellular tubulin into polymer, only stimulates tubulin phosphorylation approximately twofold in differentiated N115 cells. Incorporation of this phosphorylated tubulin species into polymer may drastically alter the interactions of microtubules with other cellular components, either directly, or through the associated proteins.

A similar polymer-dependent phosphorylation of β-tubulin has been observed (by immunoprecipitation) in two other neuroblastoma cell lines (rat B35 and B104) at levels about one-tenth that seen in N115 cells, and has tentatively been observed at even lower levels in mouse 3T3 cells (D. L. Gard, unpublished observations). This suggests that β-tubulin phosphorylation is not unique to the N115 cell line, though it may be more prominent in neuronal cells.

We have also observed in $^{32}PO_4$-incorporation into a high

molecular weight cellular phosphoprotein identified as a cellular counterpart to brain MAP-1. It is worth noting that the N115 MAP-1 species is quite sensitive to proteolysis during preparation of cell extracts. Omission of Pepstatin A resulted in cleavage of the 350,000-mol-wt species to a closely spaced doublet with a molecular weight of ~280,000 which was capable of assembling into microtubules (D. L. Gard, unpublished observations). Phosphoamino acid analysis revealed that the N115 MAP-1 contains both *o*-phosphoserine and *O*-phosphothreonine (Fig. 7*b*). Tryptic peptide analysis reveals a complex pattern of as many as 20 phosphorylation sites (D. L. Gard, unpublished observations). In similar studies, Greene et al. (42) have observed an increase in MAP-1 synthesis and phosphorylation during neural growth factor–induced differentiation and neurite outgrowth by the rat PC-12 pheochromocytoma cell line. The significance of this MAP-1 phosphorylation in N115 and PC12 cells is not known. However, the lack of colcemid and taxol sensitivity indicates that phosphorylation of MAP-1 in vivo is regulated in a manner distinct from that of β-tubulin. N115 differentiation is also accompanied by changes in phosphorylation and synthesis of numerous other proteins, to which no identity or function can currently be assigned (see Figs. 2, 3, and 7).

In summary, we have observed a differentiation-specific increase in phosphorylation of an isoform of β-tubulin in N115 cells. The sensitivity of this phosphorylation to colcemid and nocodazole and its induction by taxol indicate that β-tubulin phosphorylation is closely coupled to cellular levels of microtubule polymer. Our present data suggest that coupling of β-tubulin phosphorylation to polymer levels occurs through the action of an unidentified phosphoprotein phosphatase which discriminates between β-tubulin in monomer and polymer. Such modifications of β-tubulin and MAP-1 may play key roles in the regulation of microtubule assembly and function necessary during neuronal differentiation.

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