THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 1990 by The American Society for Biochemistry and Molecular Biology, Inc.

Vol. 265, No. 23, Issue of August 15, pp. 13949-13954, 1990
Printed in U.S.A.

Phosphorylation of a Neuronal-specific β-Tubulin Isotype*

(Received for publication, December 22, 1989)

Javier Díaz-Nido‡, Luis Serrano‡, Carlos López-Otín‡§, Joël Vandekerckhove¶, and Jesús Avila‡

From the ‡Centro de Biología Molecular, Universidad Autónoma, Canto Blanco, E-28049 Madrid, Spain

Adult rats were intracraneally injected with [^{32}P] phosphate and brain microtubules isolated. The electrophoretically purified, *in vivo* phospholabeled, β-tubulin was digested with the V8-protease and the labeled peptide purified by reversed-phase liquid chromatography. Its amino acid sequence corresponds to the COOH-terminal sequence of a minor neuronal β3-tubulin isoform from chicken and human. The phosphorylation site was at serine 444. A synthetic peptide with sequence EMYEDDEEESESQGPK, corresponding to that of the COOH terminus of β3-tubulin, was efficiently phosphorylated *in vitro* by casein kinase II at the same serine 444.

The functional meaning of tubulin phosphorylation is still unclear. However, the modification of the protein takes place after microtubule assembly, and phosphorylated tubulin is mainly present in the assembled microtubule protein fraction.

The existence of a number of different isoelectric forms of α- and β-tubulin subunits (1), greater than the actual number of tubulin genes (2–4), suggests that tubulin heterogeneity is probably due in part to posttranslational modifications of this protein. One of these modifications is phosphorylation. The *in vivo* phosphorylation of the β-tubulin subunit in differentiated neuroblastoma cells has been demonstrated (5–8); it occurs mainly on a serine residue in a specific β-tubulin isoform (5–7) and is correlated with an increased assembly of microtubules during neurite outgrowth (5). More recently, it has been shown that the phosphorylation of β-tubulin found in neuroblastoma cells is possibly due to the action of a casein kinase II-related enzyme (8). Using tryptic and CNBr cleavage, it was demonstrated that the phosphorylated residue is located close to the carboxyl terminus (8). These results are compatible with two possibilities: either the phosphorylation takes place on serine 413 in the major constitutive β_1 isoform, or on serines 444 and/or 446 in the minor neural β_3 isotubulin (see the nomenclature of Cleveland (2), or β_6 in the nomenclature of Cowan *et al.* (9)). It has recently been suggested that a β-tubulin isoform, which probably corresponds to β_3-tubulin, is that phosphorylated in neuroblastoma cells (10).

In this study, we have isolated *in vivo* phosphorylated β-tubulin from rat brain and localized the modified residue at serine 444 of the minor neural β_3-isotubulin. A synthetic peptide, corresponding to the COOH terminus of β_3-tubulin, is also efficiently phosphorylated *in vitro* by casein kinase II at serine 444. Finally, it was found that phosphorylated tubulin is mainly present in the assembled microtubule fraction.

EXPERIMENTAL PROCEDURES

Materials—Antibody against β-tubulin subunit was obtained from Amersham Corp (United Kingdom). Antibody to β_3-tubulin was obtained using a synthetic peptide containing the carboxyl-terminal sequence of β_3-tubulin as immunogen.[1] Antibody against acetylated tubulin was a kind gift of Dr. Piperno, Rockefeller University. Taxol was a kind gift from N. R. Lomax at the National Institutes of Health. ^{125}I-Protein A from *Staphylococcus aureus* was obtained from Amersham Corp. (United Kingdom).

Purification of Protein—Casein kinase II was purified from rat brain and assayed following the method of Meggio *et al.* (11).

The isolation of microtubule protein and of tubulin depleted of associated proteins has also been previously detailed (8, 12).

Cell Culture, Labeling, Extraction, and Immunoblotting—N115 mouse neuroblastoma cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and induced to differentiate by transfer to serum-free Dulbecco's modified Eagle's medium for 10 days. The cells were then washed with Dulbecco's modified Eagle's medium containing one-tenth the normal phosphate concentration and incubated overnight in the same medium, to which 500 μCi/ml $^{32}PO_4$ (HCl and carrier-free) (Amersham Corp.) had been added.

After the incubation with labeled phosphate, the cells were washed with 10 mM phosphate buffer, pH 7.0, 0.15 M NaCl. Soluble and assembled tubulin fractions were extracted *in situ* from N115 neuroblastoma cells following the procedure of Black *et al.* (13), with slight modifications. Briefly, cells attached to culture dishes were permeabilized by adding a buffer containing 0.1 M PIPES,[2] pH 6.9, 0.25% (w/v) Triton X-100, 2 M glycerol, 1 mM $MgCl_2$, 2.5 mM EGTA, 10 μg/ml aprotinin, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, 1 mM PMSF, 20 mM NaF, and 1 mM ATP. The soluble protein fraction obtained after incubation in this buffer includes unassembled microtubule protein, whereas assembled microtubule protein largely partitions with the detergent-insoluble protein fraction. The latter fraction can be extracted by resuspension of the cells in 0.1 M PIPES, pH 6.9, containing 6 mM $CaCl_2$, 10 μg/ml aprotinin, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, 1 mM PMSF, 20 mM NaF, and incubation for 30 min at 0 °C. The extracted protein was isolated in the supernatant after centrifugation for 15 min at 20,000 × *g* at 2 °C. The cold/calcium-insoluble fraction was resuspended in 20 mM Tris-HCl, pH 7.0, containing 1% sodium dodecyl sulfate and 2% β-mercaptoethanol. Aliquots of these fractions were characterized by gel electrophoresis. Following electrophoresis, the proteins were either stained in the gel with Coomassie Blue or blotted onto nitrocellulose paper (14), to be incubated with anti-β-tubulin and anti-β_3-tubulin antibodies.

To measure the amount of assembled tubulin, the above microtubule fractions, separated by electrophoresis and blotted onto paper, were first incubated with a monoclonal antibody against β-tubulin and subsequently with a polyclonal antibody to mouse IgGs raised in rabbit and *S. aureus* protein A labeled with ^{125}-iodine (Amersham Corp.). The amount of tubulin present in each fraction was calculated

* This work was supported by the Spanish Comision Asesora para la Investigación Científica y Técnica. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Present address: Departamento de Biología Funcional, Facultad de Medicina, Universidad de Orviedo, Spain.

¶ Present address: Laboratory for Genetics, State University of Ghent, Ledeganckstraat 35, B-9000 Ghent, Belgium.

[1] J. E. Domínguez, manuscript in preparation.

[2] The abbreviations used are: PIPES, 1,4-piperazinediethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; MES, 4-morpholineethanesulfonic acid; HPLC, high performance liquid chromatography; MAPS, microtubule-associated proteins.

to be proportional to the number of ^{125}I cpm measured in that fraction.

In Vivo Phosphorylation and Isolation of Tubulin Phosphopeptide—Thirty-day-old rats were anesthetized and received intracraneal injections of 1 mCi of [^{32}P]phosphate (Amersham Corp., 40 mCi/ml). After 12 h, rats were killed and the brains collected. Microtubule protein was then prepared as previously described (12), using 10 μM taxol to promote assembly in the presence of 1 mM GTP, 1 mM ATP, 20 mM NaF, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml pepstatin A, and 10 μg/ml leupeptin. The polymerized fraction was isolated after centrifugation through a cushion of 40% (w/v) sucrose in buffer A (0.1 M MES, pH 6.4, 2 mM EGTA, 0.5 mM $MgCl_2$). The resulting pellet was washed in buffer A containing 1 mM GTP, 1 mM ATP, 1 mM PMSF, 20 mM NaF, and 1 M NaCl to decrease the proportion of microtubule-associated proteins in the polymer. The polymerized protein was electrophoresed under the conditions of Laemmli on 7.5% polyacrylamide gels (15). The dried gel was autoradiographed and the labeled β-tubulin band excised and extensively digested with *S. aureus* V8 protease (3% (w/v) for 24 h at 37 °C) in 50 mM NH_4HCO_3, pH 8.0 (16). The resulting peptides were lyophylized and dissolved in 0.1% (v/v) trifluoroacetic acid. They were then loaded onto a reverse-phase HPLC column (NOVAPACK C_{18}) and fractionated using different 0–100% acetonitrile gradients (see legends to the figures), with a flow rate of 0.5 ml/min. Fractions (0.5 ml) were collected and the associated radioactivity determined by liquid scintillation counting (Cerenkov radiation). Phosphoamino-acid analysis was performed as described (8).

In Vitro Phosphorylation Assays of Synthetic Peptides—Peptides were synthesized on an automatic solid-phase peptide synthesizer (type 430A, Applied Biosystems) (Foster City, CA) and purified by reverse-phase HPLC on a NOVAPAK C_{18} column, using a Waters 501 apparatus. The peptides were lyophilized and dissolved in 0.1 M PIPES, pH 6.9, 2 mM EGTA, 5 mM $MgCl_2$, and 1 mM PMSF.

Kinase assays were performed in a final volume of 0.1 ml containing 1 mM peptide and 1 mM [γ-^{32}P]ATP, except where indicated. The samples were incubated at 30 °C and reactions terminated by adding 1 M HCl. After heating the sample for 5 min in a boiling water bath, an equal volume of 0.1% trifluoroacetic acid was added. The sample was then applied to a NOVAPAK C_{18} column equilibrated with 0.1% trifluoroacetic acid. The column was extensively washed with 0.1% trifluoroacetic acid, and the peptide was then eluted with an acetonitrile gradient. The peptide-associated radioactivity was determined by liquid scintillation counting or by Cerenkov radiation. Samples containing only casein kinase II plus ATP were used to determine the background. Counts obtained from blank samples were substracted from those obtained when the peptide was present.

In vitro phosphorylation of tubulin by casein kinase II was performed as previously indicated (8).

Other Protein-Chemical Procedures—Thin layer electrophoresis was carried out on cellulose plates (Cel 300, Merck Darmstadt Federal Republic of Germany), using 10% pyridine, 0.5% acetic acid, pH 6.3, as running buffer. Autoradiography was performed using Kodak XR film with 48-h exposure times.

Peptides were sequenced with an Applied Biosystems gas-phase sequenator (model 470A) equipped with an on-line phenylthiohydantoin amino acid analyzer (model 120 A).

RESULTS

To characterize brain phosphotubulin, an intracraneal injection of [^{32}P]phosphate into an adult rat was performed. Twelve h after injection, microtubules were isolated, in the presence of taxol and salt, to obtain a preparation of assembled tubulin with a low proportion of microtubule-associated proteins (MAPs). Fig. 1 shows that only β-tubulin and high molecular weight MAPs were phosphorylated. Phosphoamino acid analysis of the β-tubulin yielded only labeled phosphoserine (Fig. 1*b*). To localize the phosphorylated site, the labeled β-tubulin was subjected to an exhaustive *S. aureus* V8 protease digestion, and the proteolytic fragments were purified by reverse-phase HPLC. The peptide pattern obtained (Fig. 2*a*) reveals the presence of one major phosphopeptide. Since this peptide eluted close to other unlabeled peptides which could contaminate it, the phosphopeptide was further subjected to HPLC fractionation using different gradient conditions (Fig. 2*b*). The purified ^{32}P-labeled peptide was

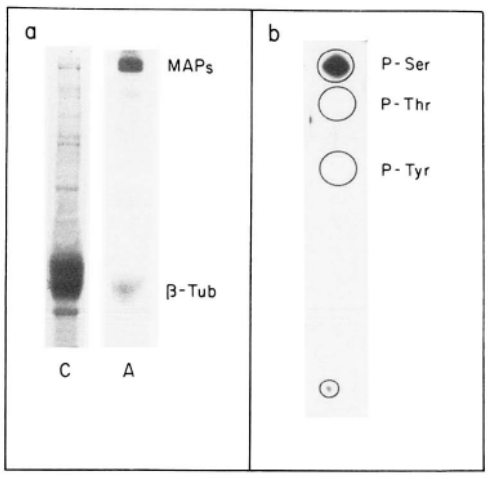

FIG. 1. ***In vivo* phosphorylation of rat brain tubulin.** *a*, rats were intracraneally injected with $^{32}PO_4$ and brain microtubules purified as indicated under "Experimental Procedures." Microtubules were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis in a 6.5% polyacrylamide gel containing 2 M urea, and the Coomassie Blue staining of the gel (*C*) and the corresponding autoradiography (*A*) are shown. Both high molecular weight MAPs and β-tubulin are phosphorylated. *b*, phosphoamino acid analysis of phosphotubulin.

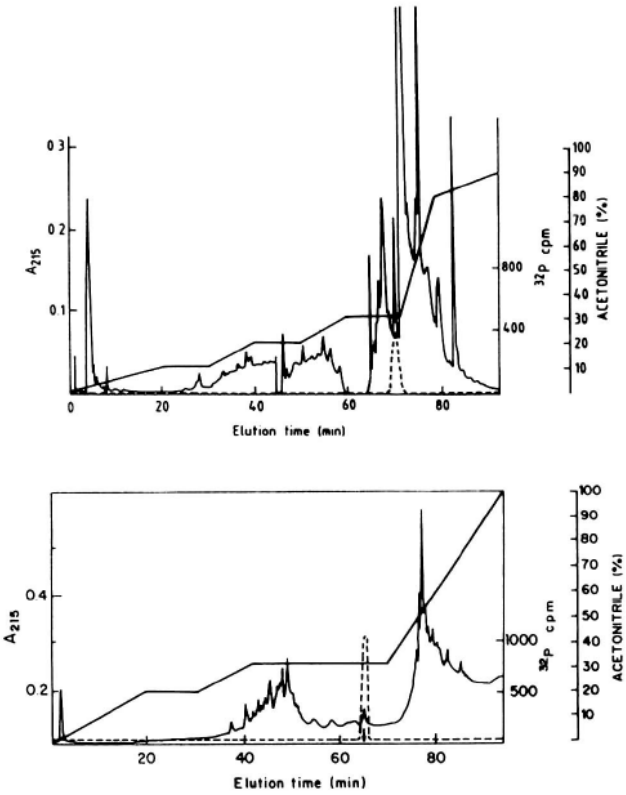

FIG. 2. **HPLC analysis of β-tubulin peptides.** *A*, phosphorylated β-tubulin band was cut out of the gel, extensively digested with *S. aureus* V8 protease, and run on an HPLC column, as described under "Experimental Procedures." Peptides were eluted with the gradient indicated in the figure at a flow rate of 0.5 ml/min. Absorbance at 215 nm (*continuous line*) and radioactive counts (*discontinuous line*) are plotted against elution time. *B*, HPLC chromatography of the β-tubulin phosphopeptide obtained after a second proteolytic digestion and fractionated with the acetonitrile gradient (in 0.1% trifluoroacetic acid) indicated in the figure at a flow rate of 0.5 ml/min. Absorbance at 215 nm (*continuous line*) and radioactive counts (*discontinuous line*) are plotted against elution time.

subjected to amino acid sequence analysis.

The derived peptide sequence MYEDDEEEXE matches the COOH-terminal sequence EMYEDDEEESESQGPK-COOH of the minor neural β_3 isoform of chick and human tubulin, as predicted from the corresponding cDNA clones (2). It is known that phosphoserine (when not modified, see for instance Ref. 18) is dephosphorylated and destroyed when removed from the peptide chain in the course of Edman degradation, thus producing a gap during sequence analysis. As it has previously been established that a β-tubulin isoform was phosphorylated at serine (see above), we conclude that the missing residue corresponding to site 444 in the β_3-tubulin sequence is the phosphorylated serine.

The cDNA sequence encoding β_3-tubulin has been determined in human, mouse, and chicken (2, 9). Different amino acids are found at residue 446: serine in human, arginine in mouse and a gap in chicken. It is not known which amino acid is present at position 446 in rat, but it does not seem to be phosphoserine, as a second phosphorylated peptide was not detected in our analyses. However, we cannot completely rule out that a presumptive serine 446 may be present adjacent to the COOH-terminal residue of the peptide MYEDDEEEXE in rat, due to an unspecific cleavage by the V8 protease after phosphorylated residues. If this is the case, this serine would have escaped our analyses, as a COOH-terminal phosphoserine would be undetectable by gas-phase sequencing. To address this problem, in addition to confirming the results of *in vivo* phosphorylation by *in vitro* assays, we synthesized a peptide corresponding to the COOH terminus of human β_3-tubulin, EMYEDDEEESESQGPK, and phosphorylated it *in vitro*. A second peptide corresponding to the region 408–428 (FTEAESNMNDLUSEYQQYQDA) of the major β-tubulin isoform(s), which contained another presumptive serine phosphorylation site, was used for comparison. *In vitro* phosphorylation was carried out using casein kinase II, as this protein kinase shows the same tubulin phosphorylation pattern as that found in neuroblastoma cells (8). Fig. 3 shows that the β_3-tubulin peptide incorporates more phosphate after incubation with ATP and casein kinase II than the other peptide. Fig. 3 also indicates an incorporation of approximately 1.3 mol of phosphate/mol of the tubulin peptide containing the COOH terminus of the minor neural isoform. Peptide phosphorylation was further tested by isolation of the phosphopeptide on thin layer electrophoresis at pH 6.5, followed by autoradiography (Fig. 4*a*). This showed one major spot corresponding to ^{32}P-labeled peptide, which is consistent with the modification of a single residue/peptide. This ^{32}P-peptide was then digested with the V8 protease and the obtained mixture was separated again by electrophoresis at pH 6.5 (Fig. 4*b*). A new spot with a higher electrophoretic mobility was now observed, which corresponded to a peptide with the sequence MYEDDEEEXE. This peptide also comigrated with the *in vivo* phospholabeled peptide in both the electrophoretic and HPLC analyses (results not shown). The second peptide released by V8 protease digestion was an unlabeled basic peptide with the sequence SQGPK.

These data are consistent with the phosphorylation of a single serine residue, corresponding to position 444 in β_3-tubulin. A similar result was obtained when the phosphorylation of tubulin in differentiated neuroblastoma cells was analyzed. In mouse neuroblastoma N115 cells, tubulin was also phosphorylated in position 444 of β_3-tubulin, as determined by sequencing the tubulin phosphopeptide isolated from differentiated cells after digestion with *S. aureus* V8 protease. The amino-terminal sequence of this peptide is MYEDDE, as that obtained from rat brain phosphotubulin.

The functional consequences of the phosphorylation of β_3-tubulin are not known, although it has been observed that phosphorylation of β-tubulin takes place after neurite extension onset in neuroblastoma cells (5, 7, 8, 10). Thus, neuroblastoma cells could be a suitable model for analysis of the

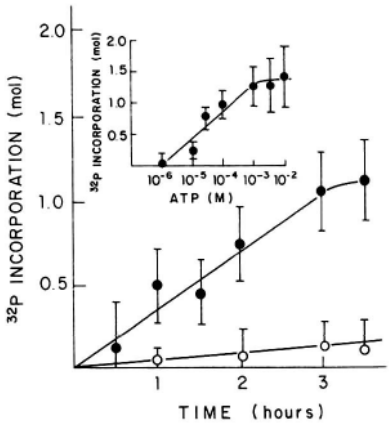

FIG. 3. **Kinetics of the *in vitro* phosphorylation of β-tubulin peptides by purified casein kinase II.** Shown is the time course of phosphorylation for the synthetic peptide (residues 435–450) from the minor neuronal β_3-tubulin isoform (*solid circles*) or that (residues 408–428) from the ubiquitous β_1-tubulin isoform (*open circles*) when phosphorylated by casein kinase II in the presence of 10 mM ATP. Standard deviations are indicated by *bars*. *Inset* shows the determination of the moles of phosphate incorporated into the synthetic peptide from the minor neuronal β_3-tubulin isoform at different ATP concentrations.

FIG. 4. **Electrophoresis of the tubulin synthetic peptide phosphorylated *in vitro* by casein kinase II.** The synthetic peptide EMYEDDEEESESQGPK was phosphorylated *in vitro* with casein kinase II as indicated under "Experimental Procedures," in the presence of 0.1 mM ATP. The phosphopeptide was further incubated in the absence (*a*) or presence (*b*) of *S. aureus* V8 protease and the labeled peptides subjected to thin layer electrophoresis in pH 6.3 buffer (10% pyridine, 0.5% acetic acid), followed by autoradiography, that is shown in the figure. To trace the electrophoretic mobility from the origin (*O*), orange G was used. The electrophoresis was stopped when the marker reached the position indicated by the *circled discontinuous line*. The major spot marked in *lane a* corresponds to the phosphorylated synthetic peptide. The minor spot with a lower electrophoretic mobility which is also found in *lane a* corresponds to a peptide lacking the amino-terminal glutamate residue of the synthetic peptide as a consequence of a low yield in the last step of the synthesis procedure. The *spots* marked in *lane b* were subjected to gas-phase sequencing as indicated in the text.

consequences of this posttranslational modification. In a pioneer work, Gard and Kirschner (5) found that tubulin modification probably occurs after the assembly of tubulin into microtubules, since drugs which prevent microtubule assembly also prevent tubulin phosphorylation. A similar conclusion may be reached from the result of the experiment indicated in Fig. 5. This figure shows that the kinetics of tubulin phosphorylation was delayed with respect to that of the net microtubule assembly which takes place during neurite extension in differentiated neuroblastoma cells. This result agrees with Gard and Kirschner's report (5) and suggests that polymerized tubulin could be a better substrate for the kinase involved in its phosphorylation than nonpolymerized tubulin. To test for this possibility, the incorporation of phosphate into tubulin at different concentrations was measured in two fractions in which tubulin is assembled to a different extent. Fig. 6*A* shows a slightly lower K_m ($0.010 > p > 0.005$) for the tubulin fraction with a higher proportion of polymerized protein with casein kinase II (the enzyme which phosphorylates tubulin in a manner similar to that found *in vivo* (8)). However, the number of moles of phosphate incorporated into tubulin was the same for both tubulin fractions (Fig. 6*B*).

To test for the *in situ* distribution of phosphorylated tubulin inside the cell, differentiated neuroblastoma cells were used. Cells were permeabilized with a nonionic detergent in a buffer which stabilizes polymerized microtubules and extracts unassembled tubulin together with other soluble proteins, and subsequently in a microtubule-disrupting buffer containing calcium, which extracts the assembled tubulin. Fig. 7 shows the characterization of the protein extracted after incubation of differentiated neuroblastoma cells with both buffers and the distribution of phosphorylated β-tubulin in the fractions containing assembled and unpolymerized tubulin. Fig. 7 indicates that phosphorylated tubulin is mainly present in the polymerized tubulin fraction inside the cell. This could be the consequence of a higher proportion of β_3 isoform in the assembled fraction, whether it is phosphorylated or not. To analyze this point, the amount of β_3 tubulin in the assembled and unassembled fractions was measured using an antibody which specifically recognizes the carboxyl-terminal region of β_3-tubulin. Fig. 8 indicates that the distribution of total β_3-tubulin is different from that of phosphorylated β_3-tubulin (compare the results of Figs. 7 and 8). As a control of the experiment of Fig. 8, the distribution of total β-tubulin using an antibody which recognizes a common epitope present in all β isoforms, and the distribution of acetylated α-tubulin (which is known to be mainly present in the assembled tubulin fraction (19)), were also tested. Fig. 8 shows that the distribution of total β-tubulin is similar to that of β_3-tubulin, while it differs from that of acetylated tubulin since this modified tubulin is almost only present in the polymerized fraction. These results could be explained by a preferential association of phosphorylated β_3-tubulin with the assembled fraction or a preferential phosphorylation of the assembled β isoform. This latter possibility is supported by the fact that colcemide (and other depolymerizing drugs) abolish β_3-tubulin phosphorylation (5).

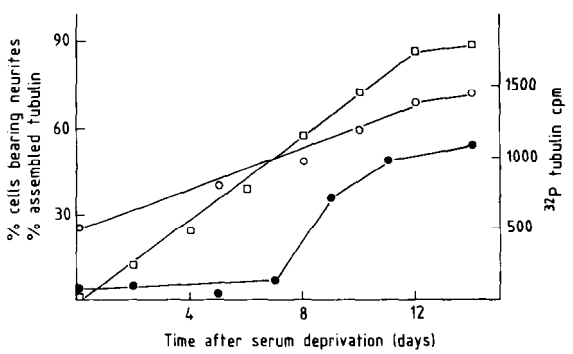

FIG. 5. **Increase in phosphorylation of β-tubulin, microtubule assembly, and neurite extension during differentiation of N115 neuroblastoma cells.** N115 neuroblastoma cells were labeled for 12 h with [^{32}P]phosphate after 0–14 days of serum withdrawal and the level of phosphorylated β tubulin (●——●) (determined after its isolation by gel electrophoresis by measuring the radioactivity associated with the protein band excised from the gel); the proportion of assembled microtubule protein (○——○) (determined as indicated under "Experimental Procedures"), and the proportion of cells bearing neurites (□——□) were measured and represented as a function of time after serum deprivation.

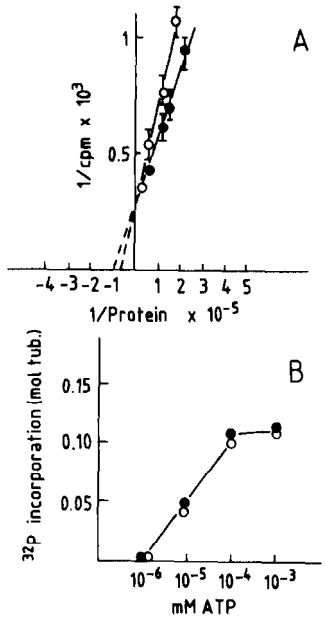

FIG. 6. **Determination of the K_m and moles of phosphate incorporated into assembled and unassembled tubulin by casein kinase II.** *A*, unassembled tubulin (0.5 mg/ml, conditions under which less than 5% tubulin is found assembled) or tubulin assembled in the presence of 10 μM taxol (0.5 mg/ml, conditions under which more than 90% tubulin is found assembled), were serially diluted and incubated with 4 μg of casein kinase II in the presence of 10^{-5} M [γ-^{32}P]ATP for 15 min at 35 °C. The reaction was stopped by adding sample electrophoresis buffer and subjected to gel electrophoresis. The inverse of the amount of labeled phosphate incorporated into β-tubulin of *versus* the inverse of the tubulin concentration is represented. The apparent K_m for taxol-assembled tubulin is 10 ± 2 μM, whereas that for unassembled tubulin is 17 ± 3 μM. *B*, aliquots of taxol-assembled and unassembled tubulin (0,5 mg/ml) were incubated with 4 μg of casein kinase II at various concentrations of ATP for 15 min at 30 °C. The reaction was stopped by adding sample electrophoresis buffer, and the samples were subjected to gel electrophoresis. After electrophoresis, the β-tubulin peptide band was excised, and its radioactivity was determined by measuring the Cerenkov radiation. The figure shows the moles of phosphate incorporated/mol of tubulin as a function of ATP concentration. Taxol-assembled tubulin is indicated by *closed circles*, and nonpolymerized tubulin by *open circles*.

DISCUSSION

The major phosphorylation of brain tubulin takes place at its minor neural β_3 isoform, as previously suggested for tubulin in neuroblastoma cells (7, 10). This minor neural tubulin isoform is only found in chordates (20, 21), and its expression may be specifically associated with a class of neuronal cells appearing with the origin of the chordates.

The *in vivo* and *in vitro* phosphorylation results indicate that the minor neural β_3-tubulin isoform is modified at a

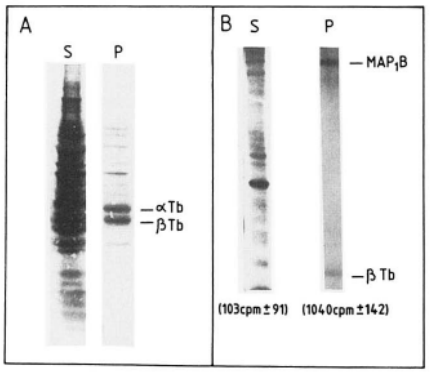

FIG. 7. **Partitioning of tubulin between soluble and microtubule assembled fractions.** Neuroblastoma N115 cells (10^8) were allowed to differentiate for 10 days in the absence of serum and then incubated overnight with [^{32}P]phosphate, as indicated under "Experimental Procedures." Extracts containing soluble (*S*) and microtubule-assembled (*P*) fractions were prepared. The extracts were divided in several aliquots and the protein present in one of these aliquots was characterized by electrophoresis in a 10% polyacrylamide gel followed by Coomassie staining of the fractionated peptides (*A*) or subjected to electrophoresis in a 6.5% polyacrylamide gel containing 2 M urea and phosphorylation of tubulin was tested by autoradiography (*B*); the counts associated with β-tubulin were determined after cutting out the β-tubulin band (after its identification by immunoblotting), and the values obtained from the gels containing the soluble (*S*) and polymerized (*P*) protein are indicated in parentheses (data represent mean values and standard deviations from five experiments). Whereas 9% of the β-tubulin radioactive counts are associated with the soluble fraction, 91% of the counts are associated with the polymerized fraction.

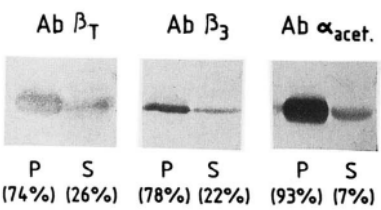

FIG. 8. **Partitioning of β_3-tubulin between the soluble and assembled microtubule fractions.** Differentiated neuroblastoma cells were processed as in Fig. 7 and, after gel electrophoresis, the proteins were blotted onto nitrocellulose paper. The paper was incubated with an antibody against β-tubulin (*AbβT*), an antibody against the carboxyl-terminal region of β_3-tubulin (*Abβ*$_3$) or a monoclonal antibody against acetylated α-tubulin (*Abα acet.*). After the first incubation, the paper was incubated with a peroxidase-conjugated goat anti-mouse or goat anti-rabbit immunoglobulin (diluted 1/1000, Tago) and developed with 4-chloronaphthol and hydrogen peroxide. The stained bands were then measured by densitometry. The proportions of each tubulin isotype present in soluble (*S*) and assembled microtubule (*P*) fractions are indicated in *parentheses*.

single serine located at position 444.

This residue, as well as its NH_2-terminal sequence seems to be extremely conserved, as it has been found in human (Hβ_4), chicken (Cβ_4), and mouse (M$_{\beta 6}$) (2, 9, 22, 23) and now also in rat. One exception is the residue 442, which is a glutamic acid in chicken, human, and, as indicated in this work, in rat, or an aspartic acid in mouse. The other exception is the residue 446 in which different amino acids, among them a serine (in human), are found. Interestingly, serine 446 is not phosphorylated, as determined by our *in vitro* studies using a synthetic peptide. From a functional point of view, the presence and modification of serine 444 must be more important than that, if any, of residue 446, as this second serine is not conserved. Furthermore, mouse β_3-tubulin is phosphorylated in the same way as rat β_3-tubulin, despite the fact that it has an arginine instead of a serine at position 446.

Since the carboxyl terminus of tubulin contains the binding site for some microtubule-associated proteins (24, 25), it is interesting to know whether the phosphorylation of β_3-tubulin might modulate the interaction of this tubulin isoform with MAPs or its ability to polymerize, in a similar way to that found for tubulin phosphorylated by calcium/calmodulin-dependent protein kinase II (26). However, it has been indicated that tubulin phosphorylated *in vitro* by casein kinase II remains associated with microtubules assembled *in vitro* (8), in contrast to tubulin phosphorylated by calmodulin kinase II (26).

The functional significance of the phosphorylation of serine 444 of β_3-tubulin is still unknown, although this modification correlates well with neurite extension in neuroblastoma cells (5, 8). As the phosphorylation of tubulin probably takes place after its association with microtubules, it may be thought that β_3-tubulin phosphorylation might favor the stabilization of the microtubules constituting the axonal scaffolding. Alternatively, β_3-tubulin may be phosphorylated after the selective stabilization of axonal microtubules.

On the other hand, we cannot rule out the presence of other phosphorylation sites on brain tubulin by other protein kinases, in case these phosphorylation events inhibit the ability of tubulin to polymerize, as our procedure to isolate *in vivo* phosphorylated tubulin from brain cytosol is based on its *in vitro* assembly. For example, it has been described that brain tubulin phosphorylated *in vitro* by Ca/calmodulin-dependent protein kinase II cannot assemble into microtubules (26). This phosphorylation occurs on serine and threonine residues within the carboxyl terminus of tubulin (26), which are different from the serine phosphorylated by casein kinase II (8) now identified as residue 444 of the β_3 isoform. Furthermore, tubulin phosphorylated *in vitro* by calmodulin kinase II may become associated with phospholipids (27). In view of the presence of tubulin in some membranous organelles including coated vesicles (28), it will be of interest to determine whether calmodulin-dependent phosphorylation of tubulin occurs *in vivo* and results in the association of the phosphorylated tubulin with these organelles.

Acknowledgments—We thank J. Van Damme for running the gas-phase sequenator, to R. Padilla for technical assistance, J. E. Domínguez for the gift of the antibody to β_3-tubulin and to Dr. G. Piperno for the gift of the antibody to acetylated α-tubulin. We also acknowledge the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment (National Cancer Institute, National Institutes of Health, Bethesda) for generously providing taxol.

REFERENCES

1. Gozes, I., and Littauer, U. Z. (1978) *Nature* **276,** 411–413
2. Cleveland, D. W. (1987) *J. Cell Biol.* **104,** 381–383
3. Villasante, A., Wang, D., Dobner, P., Dolph, P., Lewis, S. A., and Cowan, N. J. (1986) *Mol. Cell Biol.* **6,** 2409–2419
4. Wang, D. A., Villasante, A., Lewis, S. A., and Cowan, N. J. (1986) *J. Cell Biol.* **103,** 1903–1910
5. Gard, D. L., and Kirschner, M. W. (1985) *J. Cell Biol.* **100,** 765–774
6. Edde, B. C., Jeantet, C., and Gros, F. (1981) *Biochem. Biophys. Res. Commun.* **3,** 1035–1043
7. Edde, B., De Nechaud, B., Denoulet, P., and Gros, F. (1987) *Dev. Biol.* **123,** 549–558
8. Serrano, L., Díaz-Nido, J., Wandosell, F., and Avila, J. (1987) *J. Cell. Biol.* **105,** 1731–1739
9. Cowan, N. J., Lewis, S. A., Garkar, S., and Gu, W. (1987) in *The Cytoskeleton in Cell Differentiation and Development* (Maccioni, R. B., and Arechaga, J. eds) pp. 157–166, IRL Press, Oxford
10. Ludueña, R. F., Zimmermann, H. P., and Little, M. (1988) *FEBS Lett.* **230,** 142–146
11. Maggio, F., Deana, A. D., and Pinna, L. A. (1981) *J. Biol. Chem.* **256,** 11958–11967

12. Díaz-Nido, J., Serrano, L., Hernández, M. A., and Avila, J. (1990) *J. Neurochem.* **54,** 211–222
13. Black, M. A., Cochram, J. M., and Kurdyla, J. T. (1984) *Brain Res.* **295,** 255–263
14. Towbin, H., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 4350–4353
15. Laemmli, U. K. (1970) *Nature* **227,** 680–685
16. Herrmann, H., Dalton, J. M., and Wiche, G. (1985) *J. Biol. Chem.* **260,** 5797–5803
17. Wiche, G. (1989) *Biochem. J.* **259,** 1–12
18. Wang, Y., Fiol, C., De Paoli-Roach, A., Bell, A. W., Hermodson, M. A., and Roach, P. J. (1988) *Anal. Biochem.* **174,** 537–547
19. Sale, W. S., Beshase, J. C., and Piperno, G. (1988) *Cell Motil. Cytoskeleton* **9,** 243–253
20. Little, M., and Ludueña, R. F. (1985) *EMBO J.* **4,** 51–56
21. Sullivan, K. F., and Cleveland, D. W. (1984) *J. Cell. Biol.* **99,** 1754–1760
22. Sullivan, K. F., and Cleveland, D. W. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 4327–4331
23. Sullivan, K. F., Havencroft, C. J., Machlin, P. S., and Cleveland, D. W. (1986) *Mol. Cell Biol.* **6,** 4409–4418
24. Serrano, L., de la Torre, R. B., Maccioni, R. B., and Avila, J. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81,** 5989–5993
25. Serrano, L., Avila, J., and Maccioni, R. B. (1984) *Biochemistry* **23,** 4675–4681
26. Wandosell, F., Serrano, L., Hernández, M. A., and Avila, J. (1986) *J. Biol. Chem.* **261,** 10332–10339
27. Hargreaves, A., Wandosell, F., and Avila, J. (1986) *Nature* **323,** 827–828
28. Pfeffer, S. R., Drubin, D. G., and Kelly, R. B. (1983) *J. Cell Biol.* **97,** 40–47