

Tubulin Phosphorylation by Casein Kinase II Is Similar to That Found In Vivo

L. Serrano, J. Díaz-Nido, F. Wandosell, and J. Avila

Centro de Biología Molecular, Universidad Autónoma, 28049 Madrid, Spain

Abstract. Purified brain tubulin subjected to an exhaustive phosphatase treatment can be rephosphorylated by casein kinase II. This phosphorylation takes place mainly on a serine residue, which has been located at the carboxy-terminal domain of the β -subunit. Interestingly, tubulin phosphorylated by casein kinase II retains its ability to polymerize in accordance with descriptions by other authors of *in vivo* phosphorylated tubulin. Moreover, the V8 phosphopeptide patterns of both tubulin phosphorylated *in vitro* by casein kinase II and tubulin phosphorylated *in vivo* in N2A

cells are quite similar, and different from that of tubulin phosphorylated *in vitro* by Ca/calmodulin-dependent kinase II. On the other hand, we have found an endogenous casein kinase II-like activity in purified brain microtubule protein that uses GTP and ATP as phosphate donors, is inhibited by heparin, and phosphorylates phosphatase-treated tubulin. Thus it appears that a casein kinase II-like activity should be considered a candidate for the observed phosphorylation of β -tubulin *in vivo* in brain or neuroblastoma cells.

TUBULIN, the main component of microtubules, is phosphorylated *in vivo* (5–7, 9, 20, 25) and can be phosphorylated *in vitro* using different protein kinases (10, 15, 35, 38, 39).

Phosphorylation of brain tubulin was observed by intracranial injection of labeled phosphate (25) and also appears using either brain explants (6) or differentiating neuroblastoma cells (9). This phosphorylation of brain tubulin occurs only in the β -subunit at its carboxy-terminal region (7) on a serine residue (7, 9). The phosphorylated tubulin was mainly present in the assembled fraction (9).

Several kinases that phosphorylate tubulin *in vitro* have been tested. Insulin receptor-associated kinase phosphorylated tyrosine residues (15, 39), and Ca/calmodulin-dependent kinase II phosphorylated tubulin in both subunits (10, 35, 38), decreasing its ability to polymerize (35, 38). Thus, it is unlikely that these kinases are responsible for the *in vivo* phosphorylation of β -tubulin.

On the other hand, the kinase activity associated with microtubules does not appear to phosphorylate tubulin significantly (23, 30, 34). This kinase activity has two components, one cAMP dependent and another cAMP independent (30), which uses GTP or ATP as phosphate donors (14) and has a great affinity for casein (25). However, these analyses were performed without a phosphatase treatment before the phosphorylation assay in order to release phosphate already present in the substrate. Thus, if as described by Eipper (5), purified brain tubulin contains bound phosphate, it may not be a good substrate for the kinase involved in its *in vivo* phosphorylation. This kinase would have to replace the phosphate residues released by the phosphatase treatment.

We have studied tubulin phosphorylation by casein kinase

II. This kinase is present in brain (24), can use GTP or ATP as phosphate donors (13), and phosphorylates the RII subunit of the cAMP-dependent kinase associated with microtubules through the microtubule-associated protein MAP₂ (1). Furthermore, casein kinase II acts upon serine and threonine residues located in highly acidic sequences containing glutamate or aspartate residues (21); such sequences have been found at the carboxy-terminal region of both tubulin subunits (22).

We show here the similarities between the characteristics of *in vivo* phosphorylated tubulin and those of tubulin phosphorylated *in vitro* using casein kinase II, suggesting that brain tubulin may be phosphorylated *in vivo* by a casein kinase II-like activity.

Materials and Methods

Purification of Protein

Pig brain microtubule protein was prepared by temperature-dependent cycles of assembly–disassembly according to Shelanski et al. (29) and was stored as pellets at -70°C . Immediately before use, pellets were resuspended in 0.1 M 2-(*N*-morpholino) ethane sulfonic acid (MES), pH 6.4, containing 0.5 mM MgCl₂ and 2 mM EGTA (buffer A), and an additional cycle of polymerization–depolymerization was performed. Tubulin depleted of microtubule associated proteins was obtained by phosphocellulose chromatography as described by Weingarten et al. (34). The concentration of tubulin was determined at A₂₈₀ = 1.15 mg/ml.

Casein kinase II was purified from rat liver as previously described (18). Casein was purchased from Sigma Chemical Co. (St. Louis, MO) and histones were a generous gift of Dr. Angela Nieto (Centro de Biología Molecular, Universidad Autónoma).

1. *Abbreviation used in this paper:* Buffer A, 0.1 M 2-(*N*-morpholino) ethane sulfonic acid, 0.5 mM MgCl₂, 2 mM EGTA.

Casein Kinase II Assay

Casein kinase II was assayed in a final volume of 0.1 ml with a kinase/substrate ratio of 0.05 wt/wt in buffer A supplemented with 5 mM MgCl₂ and 10 μM [³²P]ATP, except when indicated. The samples were incubated for 15 min at 37°C. To determine the phosphate incorporated into protein and prevent phosphatase action, 20 mM potassium fluoride was added.

Phosphatase Treatment

Alkaline phosphatase from calf intestine (Boehringer Mannheim) was added to tubulin or microtubule protein (5.10⁻² U/μg of substrate), in 0.1 M Pipes, pH 6.9, 2 mM EDTA, 2 mM EGTA, and incubated for 30 min at 37°C. Potassium fluoride was then added to a final concentration of 10 mM and the sample was incubated with 1 mM GTP, 5 mM MgCl₂, and 10% DMSO for 30 min at 37°C. Polymerized protein was obtained by centrifugation and used as a substrate for casein kinase II.

Phosphoamino Acid Analysis

Phosphoamino acids were separated by one-dimensional chromatography on cellulose gel layers using acetic acid/pyridine/water (50:5:945) at 900 V for 60 min.

Polymerization Assay

Microtubule protein was assembled by incubation at 37°C for 30 min in buffer A in the presence of 1 mM GTP. For tubulin depleted of microtubule-associated proteins, the addition of 10% vol/vol DMSO or 10 μM taxol was required for assembly. The polymerized protein was sedimented by a 5-min centrifugation at 25°C in an airfuge (Beckman Instruments, Inc., Fullerton, CA). The polymerized and unpolymerized proteins were boiled in the presence of 1% wt/vol SDS, 2% 2-mercaptoethanol, 10% vol/vol glycerol and subjected to electrophoresis.

Gel Electrophoresis

SDS-PAGE was performed according to the procedure of Laemmli (16), and gels were stained with Coomassie Blue as indicated by Fairbanks et al. (8). Phospholabeled proteins were visualized by autoradiography of dried gels exposed to Kodak X-Omat film.

Protein IEF was performed as described by Diez et al. (4).

Tubulin Proteolysis

Limited proteolysis of tubulin with subtilisin was performed using the method of Serrano et al. (26).

Before the exhaustive tryptic digestion of tubulin, the protein was boiled for 3 min in the presence of 5 mM 2-mercaptoethanol and sedimented by centrifugation. The tubulin pellet was resuspended in 0.1 M Tris-HCl, pH 8.0, and incubated with trypsin (2% wt/wt with respect to tubulin) for 4 h at 37°C. The process was repeated twice with newly added trypsin.

Formic acid cleavage at Asp-Pro bands was performed as described by Serrano et al. (26) following the procedure of Sonderegger et al. (31).

Limited digestion of β-tubulin with *Staphylococcus aureus* V8 protease was performed as described by Cleveland et al. (3). An exhaustive digestion with the same protease was also done under the conditions described by Herrmann et al. (14).

Cell Culture and In Vivo Phosphorylation of Tubulin

N2A mouse neuroblastoma cells were grown in DME supplemented with 10% FCS, and induced to differentiate by transfer to DME without serum for 2 d.

Phosphorylated tubulin was obtained from differentiating N2A cells by a taxol-dependent procedure or by copolymerization with exogenous microtubule protein. Essentially, 50-mm culture dishes of differentiating cells were rinsed with DME containing one-tenth the normal phosphate concentration and incubated overnight in the same medium, to which 500 μCi/ml ³²PO₄ (HCl and carrier free) (Amersham Corp., Arlington Heights, IL) had been added. Cells were then washed with 10 mM phosphate buffer, pH 7.2, 150 mM NaCl (PBS), and gently scraped from the culture dish with a rubber policeman into 1 ml of cold PBS, centrifuged for 2 min at 1000 g and resuspended in 200 μl of buffer A supplemented with 1 mM PMSF, 20 mM KF, and 1 mM ATP. The cells were then homogenized in the presence of brain microtubule protein (2 mg/ml). After centrifugation in a Beck-

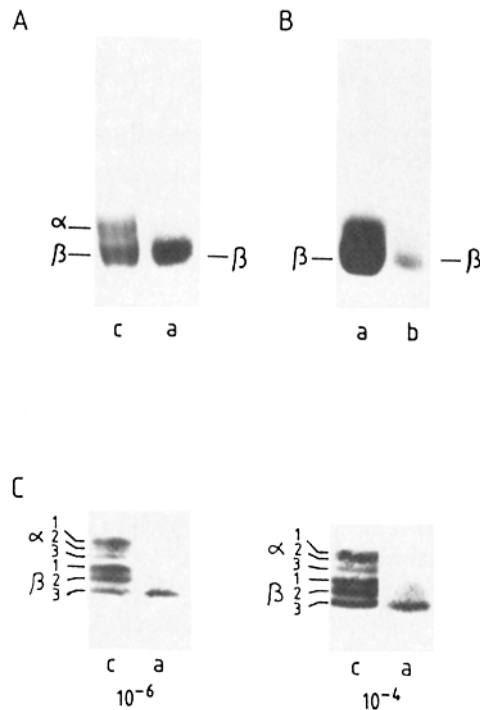


Figure 1. Phosphorylation of tubulin with casein-kinase II. (A) 100 μg of purified brain tubulin (2 mg/ml) were incubated with 3 μg of casein kinase II in buffer A plus 5 mM MgCl₂ and 10⁻⁵ M [³²P]ATP. The Coomassie Blue pattern (lane c) and the corresponding autoradiography (lane a) of the protein subjected to SDS-PAGE in a 7.5% polyacrylamide slab gel are shown. (B) 100 μg of purified brain tubulin (2 mg/ml) were incubated with or without 3 μg of casein kinase II under the conditions described in A. The autoradiographies corresponding to tubulin incubated with the kinase (lane a) or without the kinase (lane b) are shown. Lane a (similar to that of A) is highly overexposed in order to show the endogenous phosphorylation of tubulin in lane b. (C) 100 μg of purified brain tubulin (2 mg/ml) were phosphorylated with casein kinase II in the presence of 10⁻⁶ or 10⁻⁴ M [³²P]ATP. The Coomassie Blue patterns (lane c) and the corresponding autoradiographies (lane a) of the protein subjected to IEF are shown.

man airfuge (5 min at 100,000 g), the collected supernatant was polymerized with 1 mM GTP in the presence or in the absence of 10 μM taxol at 37°C for 15 min, layered onto a cushion of 40% wt/vol sucrose in buffer A, and centrifuged in a Beckman airfuge (7 min at 100,000 g). The resulting pellets were resuspended in 100 μl of buffer A plus 10 μM taxol, 1 mM GTP, 1 mM PMSF, 20 mM KF, and 1 M NaCl, incubated for 10 min at 37°C, centrifuged on the sucrose buffer, and subjected to electrophoresis. The dried gel was autoradiographed and the labeled tubulin band was excised and digested with *S. aureus* V8 protease (3).

Extensive *S. aureus* V8 protease digestions of the labeled β-tubulin bands were also performed as described by Herrmann et al. (14). The resulting phosphopeptides, in 0.1% vol/vol trifluoroacetic acid, were applied to a reverse-phase HPLC column (NOVAPACK-18) and fractionated using a 0–80% acetonitrile gradient in 0.1% trifluoroacetic acid with a flow rate of 0.5 ml/min. Fractions of 0.5 ml were collected and the associated radioactivity determined by measuring Cerenkov radiation. Alternatively, the V8 phosphopeptides were analyzed on a two-dimensional system using a thin-layer cellulose chromatography plate (20 × 20). The first dimension was run in acetic acid/formic acid/water (1.5:0.5:8) and the second dimension in chloroform/methanol/ammonia (2:2:1).

Immunoprecipitation Analysis

Immunoprecipitation of tubulin from labeled cell extracts or porcine brain microtubule protein after phosphocellulose chromatography (34) was per-

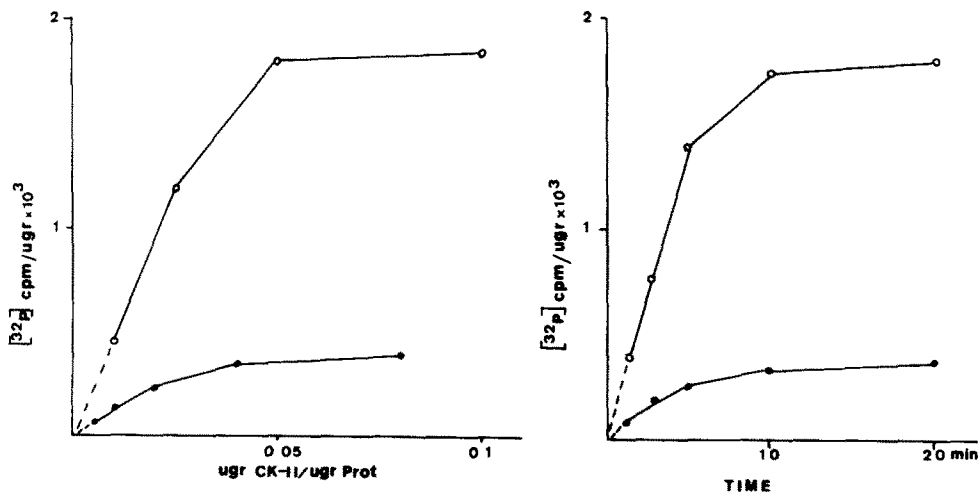


Figure 2. Kinetics of phosphorylation of tubulin or casein by casein-kinase II. (Left) Casein (1 mg/ml; open circles) or tubulin (1 mg/ml; solid circles) were incubated with different concentrations of casein kinase II in buffer A plus 5 mM MgCl₂ and 10⁻⁴ M [γ -³²P]ATP for 15 min at 37°C. The protein-associated radioactivity is determined by Cerenkov radiation. The plot of labeled phosphate incorporated into protein (cpm/ μ g) versus the enzyme/substrate ratio is shown. (Right) Casein (open circles) or tubulin (solid circles) were incubated as described above with casein kinase II, maintaining a constant enzyme/substrate ratio (0.05) and varying the incubation time (0 to 20 min). The plot of labeled phosphate incorporated into protein (cpm/ μ g) versus the reaction time (min) is shown.

formed essentially as described by Gard and Kirschner (9), using a monoclonal antibody against β -tubulin (No. 357, Amersham Corp.).

Results

Phosphorylation of Tubulin by Casein Kinase II

Phosphocellulose-purified tubulin was phosphorylated by casein kinase II under the standard conditions described in Materials and Methods.

In Fig. 1 A, the electrophoretic pattern and the corresponding autoradiography of phosphorylated tubulin are shown. It was observed that casein kinase II phosphorylated almost exclusively the β -subunit of tubulin. Fig. 1 B shows that phosphorylation of β -tubulin without added enzyme (lane b) is almost negligible compared with the phosphorylation of β -tubulin in the presence of casein kinase II (lane a). However, this endogenous phosphorylation of β -tubulin is completely inhibited by heparin, indicating that this is due to traces of a casein kinase II-like activity associated with brain microtubule protein (discussed below).

When phosphorylated tubulin was subjected to IEF, one β -isoform contained almost all the labeled phosphate. An increase in this isoform was also noted at higher concentrations of ATP (from 20% at 10⁻⁶ M ATP to 31% at 10⁻⁴ M ATP, as determined by densitometric scanning of Coomassie Blue-stained protein), with respect to the total amount of β -subunit, without the appearance of new, more acidic, labeled bands. This may be in accordance with the fact that a specific β -tubulin isoform (30% of β) is phosphorylated in N115 neuroblastoma cells (9).

To determine whether the phosphorylation of tubulin is physiologically relevant, we have followed the suggestions made by Greengard and co-workers (19) in determining both

the enzyme/substrate ratio (Fig. 2, right) and the reaction time (Fig. 2, left) required for reaching a plateau in the incorporation of phosphate to tubulin or casein. Although the extent of phosphorylation of both substrates was different, the plateau was reached at the same enzyme/substrate ratio (0.04) and with the same reaction time (10 min).

The K_m was 20 μ M for tubulin and 2 μ M for casein (Fig. 3 A). These values were determined using different substrate concentrations (from 10⁻⁶ to 10⁻⁵ M) with fixed enzyme (2.10⁻² μ g/ml) and ATP (10⁻⁴ M) concentrations and a reaction time of 15 min.

Since purified brain tubulin contained bound phosphate as described by Eipper (5, 7), the phosphorylation assay was also performed after treatment with alkaline phosphatase, which removes endogenous phosphate residues. In this way, we have found that phosphorylation of phosphatase-treated tubulin by casein kinase II (Fig. 3 B) results in the incorporation of 0.12 mol of phosphate per mol of tubulin.

Moreover, whereas casein kinase II added extra phosphate to phosphatase-treated tubulin, Ca/calmodulin-dependent kinase II showed no differences in the number of moles of phosphate incorporated into tubulin or in the degree of phosphorylation after the phosphatase treatment (data not shown). Thus, it appears that only casein kinase II can replace the phosphatase-released phosphate present in purified brain tubulin.

While casein kinase II phosphorylated untreated tubulin on both serine and threonine residues, with a slight bias to serine (2 mol of phosphoserine per 1.5 mol of phosphothreonine) (Fig. 4, lane T-), phosphatase-treated tubulin was phosphorylated predominantly on serine (Fig. 4, lane T+). Thus, phosphatase treatment released phosphate from serine residues that could be reincorporated by incubation with casein kinase II.

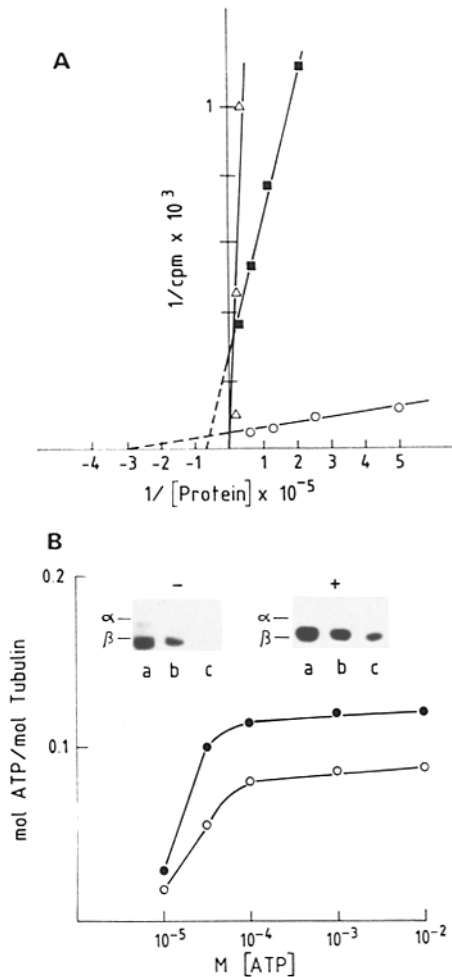


Figure 3. Determination of the K_m and moles of phosphate incorporated into tubulin by casein-kinase II. (A) 100 μ g of tubulin (square), casein (circle), or histones (triangle) at different concentrations (from 10^{-6} to 10^{-5} M), were incubated with 3 μ g of casein kinase II in the presence of 10^{-4} M [γ - 32 P]ATP for 15 min at 37°C. The reaction was stopped and the incorporation of phosphate was determined. The labeled phosphate incorporated into protein versus the inverse of the substrate concentration is represented. (B) 50 μ l aliquots of purified brain tubulin (5 mg/ml) at various concentrations of ATP were treated with alkaline phosphatase as described in Materials and Methods. After polymerization in the presence of 10% DMSO and 1 mM GTP, the polymerized protein was resuspended in buffer A and incubated with casein kinase II (at an enzyme/substrate ratio of 0.05) for 15 min at 37°C. Each aliquot was subjected to SDS-gel electrophoresis and the relevant bands were excised from the gel and their radioactivity measured by Cerenkov radiation and converted to concentrations of ATP. Experiments were also done in parallel, omitting treatment with phosphatase. The figure shows the number of moles of phosphate incorporated per mole of tubulin as a function of the concentration of ATP for both phosphatase-treated (solid circles) or untreated (open circles) tubulin. Inset shows the autoradiography corresponding to 10^{-5} M ATP (lane a), $5 \cdot 10^{-5}$ M ATP (lane b) and 10^{-4} M ATP (lane c) for both phosphatase-treated (+) or untreated (-) tubulin.

Localization of Phosphorylated Residues

Eipper indicated that tubulin was phosphorylated *in vivo* in a tryptic peptide of ~ 50 amino acids located in the carboxy-terminal region of β -tubulin (5). Thus, an exhaustive tryptic digestion of *in vitro* phosphorylated tubulin was performed

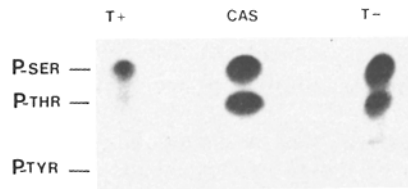


Figure 4. Phosphoamino acid analysis of tubulin phosphorylated by casein kinase II. Phosphoamino acid analysis (performed as indicated in Materials and Methods) of phosphatase-treated tubulin (T+), non-treated tubulin (T-) and casein (CAS) phosphorylated by casein kinase II.

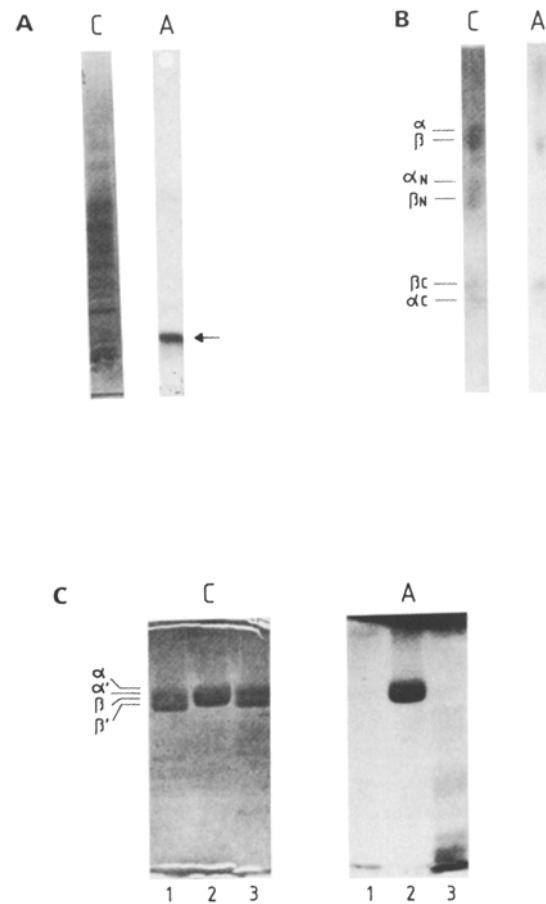


Figure 5. Localization of the phosphorylated residue at the COOH-terminal of β -tubulin. (A) Tubulin phosphorylated by casein kinase II was digested with trypsin as described in Materials and Methods. The figure shows the Coomassie Blue pattern (lane C) and the corresponding autoradiography (lane A) of the tryptic phosphopeptides separated by SDS-PAGE on a 10–20% gradient slab gel containing 8 M urea. (B) Tubulin phosphorylated by casein kinase II was cleaved by formic acid. The figure shows the Coomassie Blue pattern (lane C) and the corresponding autoradiography (lane A) of the tubulin fragments: β_N (amino acids 1–306, 30–306), β_C (amino acids 307–455), α_N (amino acids 1–306), and α_C (amino acids 307–453) separated by SDS-PAGE on a 12.5% slab gel. (C) Subtilisin-digested tubulin was incubated with casein kinase II (lane 1) in parallel with undigested tubulin (lane 2). Tubulin phosphorylated with casein kinase II was followed by a subtilisin digestion (lane 3). The figure shows the Coomassie Blue pattern (gel C) and the corresponding autoradiography (gen A) of tubulin and its fragments separated by SDS-PAGE on a 7.5% slab gel.

to determine the peptide or peptides phosphorylated in phosphatase-treated tubulin by casein kinase II. This showed only one tryptic phosphopeptide with an apparent molecular mass of 4 kD (Fig. 5 A).

To localize this phosphopeptide in the tubulin molecule, formic acid digestion of phosphorylated tubulin was performed (Fig. 5 B). Formic acid cleaves both tubulin subunits into two principal fragments, one comprising the amino-terminal region (α_N and β_N) and the other comprising the carboxy-terminal region (α_C and β_C) (26). Fig. 5 B shows that only the carboxy-terminal fragment of β -tubulin (β_C), comprising amino acids 307–455 as numbered by Ponstingl et al. (22), was phosphorylated.

To localize more precisely the casein kinase II site, phosphorylated tubulin was digested with subtilisin, which removes a fragment from the COOH terminal of both tubulin subunits comprising amino acids 418–453 in α -subunit and 418–455 in β subunit (17, 26). We found no phosphate residues in subtilisin-digested tubulin, indicating that the phosphorylated residue was contained in the COOH-terminal domain (amino acids 418–455) of β -tubulin (Fig. 5 C). Moreover, tubulin previously digested by subtilisin was not a substrate for casein kinase II, confirming the previous results (Fig. 5 C).

In view of these data, the phosphorylated residue must be located in the subtilisin COOH-terminal fragment (amino acids 418–455) of β -tubulin. Since this peptide has no lysine or arginine residues, and the nearest basic residue is lysine 402, the tryptic phosphopeptide shown in Fig. 5 A must therefore be the one comprising amino acids 402–455.

Cyanogen bromide digestion was performed to further localize the phosphorylated residues. Upon cleaving the tryptic phosphopeptide (amino acids 402–455) with cyanogen bromide, the phosphorylated residue was found in a peptide of M_r 1,500 comprising amino acids 416–425 (Fig. 6). Consequently, the residue of β -tubulin phosphorylated by casein kinase II after phosphatase treatment should be serine 423 (as numbered by Ponstingl et al., 22). However, Sullivan et al., have recently described a new neuronal specific β -tu-

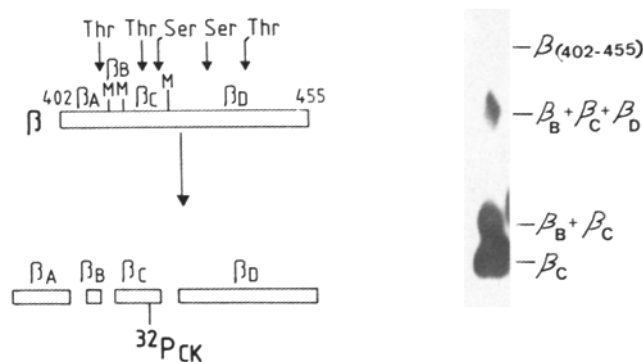


Figure 6. Determination of the phosphorylated residue. The tryptic phosphopeptide of β -tubulin phosphorylated by casein kinase II (Fig. 5 A) was further cleaved with cyanogen bromide, and the fragments were separated by SDS-PAGE on a 20% slab gel with 8 M urea. Ovalbumin, lysozyme, cytochrome *c*, insulin, glucagon, and the β -tubulin tryptic phosphopeptide were used as molecular mass markers. The figure shows the autoradiography of the cyanogen bromide fragments and a scheme that indicates the position of the possible phosphorylation site.

bulin isotype in chicken (33) and man (32), which has a novel serine at its carboxy terminus. This serine residue is contained in a cyanogen bromide peptide not present in other isoforms of a size similar to the fragment described above. Thus, if pig contains a similar isotype, we cannot determine whether phosphorylation takes place in this residue or in both.

Polymerization of In Vitro Phosphorylated Tubulin

In vivo phosphorylated tubulin is present mainly in the polymerized fraction (9). Interestingly, phosphorylation of tubulin by casein kinase II does not inhibit assembly (see Table I), thus differing from the effect found with other protein kinases, especially with Ca/calmodulin-dependent kinase II (35).

Furthermore, we find that a dilute solution of tubulin previously polymerized in the presence of taxol and GTP is a better substrate for casein kinase II than the same preparation of unpolymerized tubulin (Fig. 7). Thus, it appears that phosphorylation of tubulin by casein kinase II occurs preferentially in the polymerized form.

Comparison of In Vivo Phosphorylated β -Tubulin in Neuroblastoma Cells and β -Tubulin Phosphorylated In Vitro with Casein Kinase II

N2A neuroblastoma cells were induced to differentiate after 2 d of incubation in serum-depleted medium. When neurites were clearly visible, cells were incubated for 12 h in DME medium supplemented with labeled phosphate.

The phosphorylated β -tubulin was obtained from the assembled fraction as described in Materials and Methods (Fig. 8 A) and excised from the gel. The isolated protein was digested with *S. aureus* V8 protease, and its digestion pattern was compared with those of phosphatase-treated β -tubulin

Table I. Proportion of Phosphorylated Tubulin in Polymerized and Unpolymerized Fractions

Sample	cpm	Tubulin μ g	Ratio cpm/ μ g
Initial sample	1,774	200	8.87
First supernatant	225	40	5.60
First pellet	1,549	160	9.68
Cold-resistant pellet	922	48	19.20
Second supernatant	138	44	3.13
Second pellet	483	68	7.10

30 μ l of tubulin (2 mg/ml) in buffer A containing 30% sucrose was previously treated with alkaline phosphatase and then phosphorylated with casein kinase II (at an enzyme/substrate ratio of 0.05) and 10^{-5} M [γ - 32 P]ATP (conditions under which ~ 0.03 mol of phosphate are incorporated per mol of tubulin). The sample was then centrifuged in a Beckman airfuge at maximum speed for 3 min at 4°C. The supernatant was then mixed with 70 μ l of two-cycled microtubule protein (1 mg/ml) in the presence of 1 mM GTP and 20 mM KF. The sample was incubated for 15 min at 37°C and the polymerized fraction separated from the unpolymerized by centrifugation. The polymerized fraction was subjected to a further cycle of temperature-dependent assembly-disassembly. Due to the presence of 1 mM GTP, no additional incorporation of 32 P into tubulin took place during the assembly process. Samples of each fraction were analyzed by SDS-PAGE after carboxymethylation (to separate α from β tubulin) and the radioactivity associated with β tubulin was determined by counting Cerenkov radiation of the excised band of the gel. The table shows the radioactive counts associated with β tubulin, the amount of tubulin, and the ratio (specific activity) calculated for each fraction.

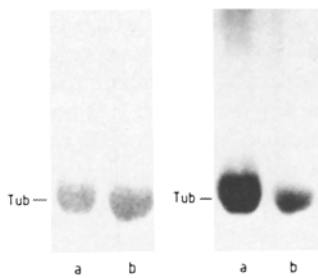


Figure 7. Preferential phosphorylation of polymerized β -tubulin. A dilute solution of phosphatase-treated tubulin (1 mg/ml) was polymerized in the presence of 10 μ M taxol and 1 mM GTP for 15 min at 30°C. The polymerized tubulin was then isolated by centrifugation and resuspended in buffer A containing 2 μ M taxol.

This fraction was incubated with casein kinase II as indicated in Materials and Methods. Unpolymerized phosphatase-treated tubulin was also used as a control. (Right) the Coomassie Blue staining pattern of both samples, polymerized (lane a) and unpolymerized (lane b) tubulin. (Left) the corresponding autoradiographies.

phosphorylated by casein kinase II or Ca/calmodulin-dependent kinase II (Fig. 8 B). The digestion pattern of in vivo phosphorylated β -tubulin was similar to that of β -tubulin phosphorylated by casein kinase II, and was clearly different from that of β -tubulin phosphorylated by Ca/calmodulin-dependent kinase II. Similar results were obtained when the comparison was performed using the cyanogen bromide cleavage patterns of the phosphorylated proteins (data not

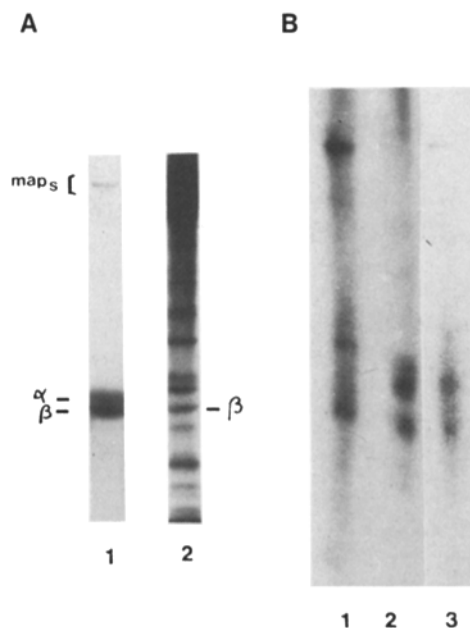


Figure 8. Comparison between β -tubulin phosphorylated in vivo and β -tubulin phosphorylated by casein-kinase II or Ca/calmodulin-dependent kinase II. (A) Differentiating N2A neuroblastoma cells, labeled as indicated in Materials and Methods, were homogenized, copolymerized with brain microtubule protein, and fractionated on a 5–15% polyacrylamide gel. The figure shows the Coomassie Blue pattern (lane 1) and the corresponding autoradiography (lane 2). Arrows indicate the positions for α and β tubulin, microtubule-associated proteins and the phosphorylated β -tubulin. (B) Comparison of the *S. aureus* V8 phosphopeptide maps of in vivo phosphorylated β -tubulin in differentiating neuroblastoma cells (lane 3) and β -tubulin phosphorylated in vitro by casein kinase II (lane 2) or Ca/calmodulin-dependent kinase II (lane 1).

shown). For a further comparison, both in vivo phosphorylated β -tubulin and β -tubulin phosphorylated in vitro by casein kinase II were subjected to an extensive V8 digestion. This protease was used as it fundamentally cleaves acidic residues, and the COOH-terminal region is an acid-rich region. The V8 peptides separated by fingerprint analysis (data not shown) or reverse-phase HPLC (Fig. 9) show patterns for tubulin phosphorylated in vivo or in vitro that are essentially identical.

Thus, it appears that β -tubulin is phosphorylated both in differentiating neuroblastoma cells and in vitro (using casein kinase II) in the same site, whereas Ca/calmodulin-dependent kinase II phosphorylated β -tubulin in other residues in the carboxy terminus of β -tubulin.

Association of a Casein Kinase II-like Activity with Purified Brain Microtubule Protein

Since our results indicated that casein kinase II is responsible for in vivo phosphorylation of β -tubulin, we studied the occurrence of a related activity in purified brain microtubule protein.

Several authors have described a microtubule-associated kinase activity similar to casein kinase II, in that it uses both ATP and GTP as phosphate donors and is independent of cAMP (14, 30). We therefore performed phosphorylation assays of brain microtubule protein purified by three assembly-disassembly cycles using ATP or GTP, either in the presence or absence of heparin, which specifically inhibits casein kinase II (13). We have also performed these assays using microtubule protein previously treated with alkaline phosphatase.

The results shown in Fig. 10 (using ATP as phosphate donor) indicate that microtubule-associated proteins are the major substrates for endogenous kinase activities. Interestingly, a kinase activity phosphorylated β -tubulin (identified by immunoprecipitation, data not shown) at its carboxy terminus (as verified by formic acid or cyanogen bromide cleavage, data not shown). This phosphorylation was only clearly apparent when the protein had previously been treated with phosphatase, and was inhibited by heparin. Similar results were obtained when GTP rather than ATP was used (data not shown), suggesting that a kinase activity similar to casein kinase II was associated with purified brain microtubule protein.

Discussion

Phosphorylation of tubulin takes place in its β -subunit in differentiating neuroblastoma cells and appears to be associated with neurite outgrowth (9). This β -subunit has previously been found to contain bound phosphate when purified from brain (5–7).

The kinase responsible for this in vivo phosphorylation of tubulin must comply with the following characteristics. (a) The kinase must have a high specific activity for tubulin. (b) In vitro phosphorylation must replace the endogenous phosphate bound to tubulin when it is removed by an exhaustive phosphatase treatment. (c) In vitro phosphorylation of tubulin must occur in the β -subunit, and this phosphorylated subunit must appear as a single band in IEF. The β -subunit must be preferentially phosphorylated on a serine residue, which must be located at its carboxy terminus (5–7, 9). (d) In vitro

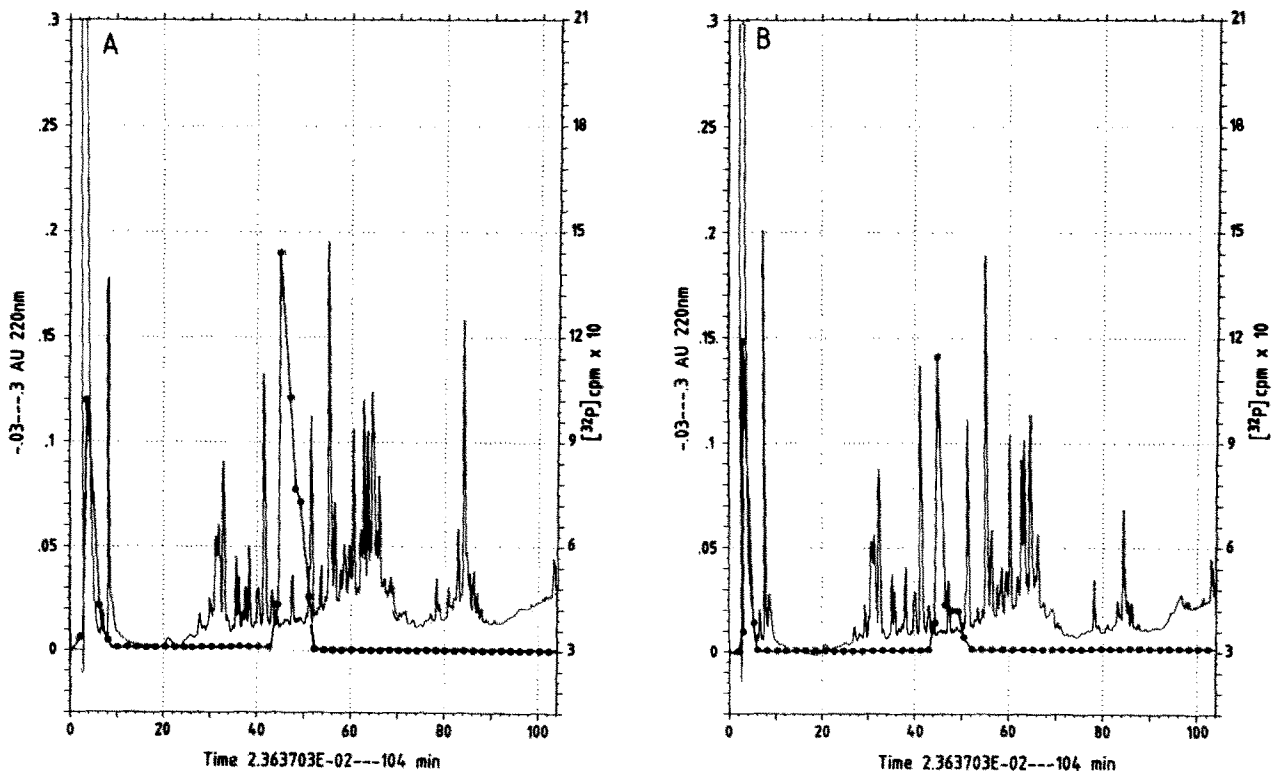


Figure 9 HPLC analysis of phosphopeptides from both in vivo phosphorylated β -tubulin and β -tubulin phosphorylated in vitro by casein kinase II. Samples similar to those described in Fig. 8 were extensively digested with *S. aureus* V8 protease and run on reverse-phase HPLC columns as described in Materials and Methods. BSA was also digested and its peptides used as position markers (continuous line). Radioactive counts associated with phosphorylated tubulin peptides are indicated by solid circles. Absorbance at 220 nm or radioactive counts are plotted versus elution time. *A* corresponds to the peptides from β -tubulin phosphorylated in vitro by casein kinase II, and *B* shows the peptides from in vivo phosphorylated β -tubulin in differentiating neuroblastoma cells.

phosphorylation of tubulin should not inhibit its polymerization (9).

Our results show that casein kinase II phosphorylates tubulin at its β -subunit with kinetics similar to that of its preferred substrate, casein, although it has a higher K_m value for β -tubulin.

Casein kinase II can replace the phosphoserine present in purified brain β -tubulin (5, 7) when this phosphate is previously removed by phosphatase, which is not the case when using Ca/calmodulin-dependent kinase II.

It has also been found that only one β -tubulin isotype is preferentially phosphorylated by casein kinase II. This phos-

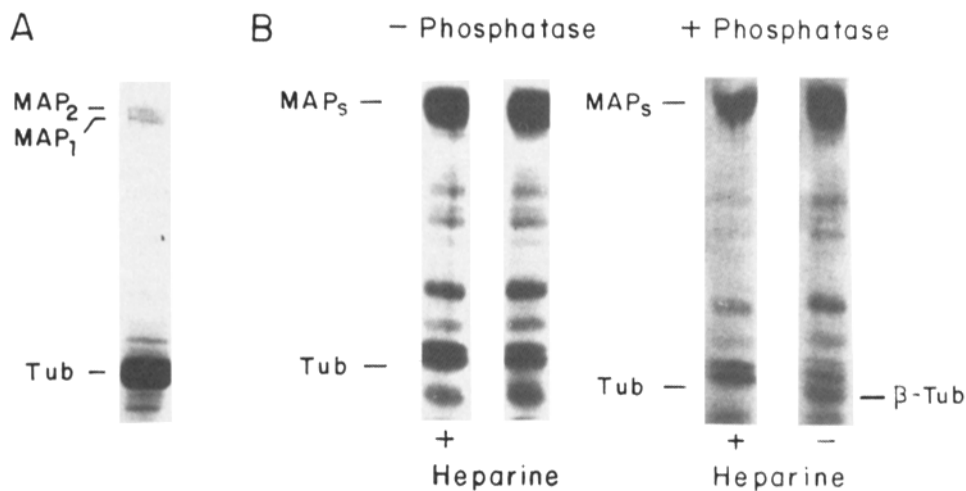


Figure 10. Casein-kinase II-like activity associated with microtubules. 100 μ l of purified microtubule protein (2 mg/ml) were treated with alkaline phosphatase (+) and phosphorylated in the presence of 10^{-5} M $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ using the endogenous kinase activities. This was done in parallel with microtubule protein not treated with phosphatase (-). (*A*) The figure shows the Coomassie Blue-stained electrophoretic pattern of the microtubule protein used in the experiment and the positions of tubulin (t), MAP_2 , and MAP_1 . (*B*) The left lane

of each pair corresponds to background phosphorylation not due to a casein kinase-like activity (assays performed in the presence of 10^{-5} M heparin) (+) while the right lane corresponds to assays performed in the absence of heparin (-). The position of tubulin (*Tub*), microtubule-associated proteins (*MAPs*), and that of phosphorylated β -tubulin subunit (*β -Tub*) are indicated.

phorylation takes place on a serine residue located at the carboxy terminus of β -tubulin. By cyanogen bromide peptide mapping, we have identified serine number 423 (Ponstingl numeration) of β -tubulin as the possible phosphorylation site. Nevertheless, we cannot discount that phosphorylation can also take place in another serine residue exclusive to the carboxy terminus of a neuronal-specific β -tubulin isotype that has been described in chicken and man (32, 33) and may be present in pig.

We have found that both β -tubulin labeled in differentiating N2A neuroblastoma cells and β -tubulin phosphorylated *in vitro* by casein kinase II are phosphorylated in the same peptide, as shown by extensive V8 digestion and HPLC or fingerprint analysis of the resulting peptides. On the other hand, their V8 phosphopeptide maps are clearly different from that of tubulin phosphorylated by Ca/calmodulin-dependent kinase II.

In vitro phosphorylation of tubulin by casein kinase II does not inhibit assembly, consistent with the fact that *in vitro* phosphorylated tubulin is present in the assembled fraction. Moreover, polymerized tubulin is a better substrate for casein kinase II than unpolymerized tubulin.

It had been suggested that casein kinase II is not associated with brain microtubule protein, because no decrease in phosphate incorporation was found in the presence of 1 mM calcium (which is an inhibitor of this kinase [14]). However, we have found a casein kinase II-like activity in brain microtubule protein purified by three cycles of assembly-disassembly. This discrepancy might be explained by the fact that calcium is not a very specific inhibitor for casein kinases (13). Thus, we have succeeded in identifying this activity using heparin, which is a much more specific inhibitor of casein kinase II than calcium (13). Furthermore, in our hands, a slight inhibition by calcium of this endogenous kinase has been observed (data not shown). In microtubule preparations pretreated with phosphatase, β -tubulin is phosphorylated by this endogenous casein kinase II-like activity. This *in vitro* phosphorylated β -tubulin has the same properties as tubulin phosphorylated by purified casein kinase II or *in vivo* phosphorylated β -tubulin. On the other hand, the existence of an endogenous kinase activity that uses GTP as phosphate donor and is able to phosphorylate tubulin must be taken into account when one uses three-cycled microtubule protein polymerized with 1 mM GTP. This may explain the differences found by some authors (23), who indicate that tubulin purified by cycles of assembly-disassembly (29) is not as good a substrate for endogenous kinases as tubulin purified by noncycling methods (i.e., see Weisenberg [37]), since tubulin could have already been phosphorylated after two cycles of assembly-disassembly by a microtubule-associated casein kinase II-like activity.

Finally, it is noteworthy that casein kinase II and Ca/calmodulin-dependent kinase II phosphorylate different residues at the carboxy terminus of tubulin, a domain of tubulin that is involved in the regulation of polymerization (17, 26) and in the interaction with microtubule-associated proteins (27, 28). Whereas tubulin phosphorylated by casein kinase II is present in the assembled fraction, the phosphorylation of tubulin by Ca/calmodulin-dependent kinase II decreases its ability to polymerize and to bind to microtubule-associated proteins (35) and enhances its association to phospholipid vesicles (12). In this context, it has been suggested that

phosphorylation by casein kinase II could result in a stable modification of proteins (13). This would support the proposed role for *in vivo* phosphorylation of β -tubulin in microtubule assembly and stabilization during neurite outgrowth in differentiating neuroblastoma cells (9) and might explain the occurrence of phosphoserine in purified brain tubulin (5, 7). On the other hand, phosphorylation of tubulin by Ca/calmodulin-dependent kinase II might have a regulatory role on the association of tubulin with certain cellular membranes, such as coated vesicles, synaptic vesicles, or postsynaptic densities (11, 12, 20). Thus, it appears that phosphorylation of tubulin at its carboxy terminus by different kinases may be a mechanism to regulate its assembly into microtubules and its interaction with other cellular organelles.

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Note Added in Proof: In a recent work (1986, *Eur. J. Biochem.*, 160:239-244) it has been shown that the sequence SEEEAEE is an optimal sequence for casein kinase II. This sequence is similar to that of a specific β -isotubulin (SEEDAEE) (32, 33).

References

1. Carmichel, F. D., L. R. Grahler, M. S. Allen, and G. E. Krebs. 1982. Type II regulatory subunit of cAMP dependent protein kinase. *J. Biol. Chem.* 257:10440-10445.
2. Deleted in proof.
3. Cleveland, D. W., S. G. Fisher, M. W. Kirschner, and U. K. Laemmli. 1977. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. *J. Biol. Chem.* 252:1102-1106.
4. Diez, J. C., M. L. Little, and J. Avila. 1984. Quantitative determination, isolation and characterization of pig lung tubulin. *Biochem. J.* 219:277-285.
5. Eipper, B. A. 1972. Rat brain microtubule protein: purification and determination of covalently bound phosphate and carbohydrate. *Proc. Natl. Acad. Sci. USA.* 69:2283-2287.
6. Eipper, B. A. 1974. Rat brain tubulin and protein kinase activity. *J. Biol. Chem.* 249:1398-1406.
7. Eipper, B. A. 1974. Properties of rat brain tubulin. *J. Biol. Chem.* 249:1407-1416.
8. Fairbanks, G., N. C. Steck, and D. F. Wallach. 1981. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry.* 10:2606-2617.
9. Gard, L. A., and M. W. Kirschner. 1985. A polymer-dependent increase in phosphorylation of β -tubulin accompanies differentiation of mouse neuroblastoma cell line. *J. Cell Biol.* 100:764-774.
10. Goldenring, R. J., B. González, J. S. McGuire, Jr., and R. J. De Lorenzo. 1983. Purification and characterization of a calmodulin-dependent kinase from rat brain cytosol able to phosphorylate tubulin and microtubule-associated proteins. *J. Biol. Chem.* 258:12632-12640.
11. Goldenring, R. J., J. S. McGuire, Jr., and R. J. De Lorenzo. 1984. Identification of the major post-synaptic density protein as homologous with the major calmodulin-binding subunit of a calmodulin-dependent protein kinase. *J. Neurochem.* 42:1077-1084.
12. Hargreaves, A., F. Wandosell, and J. Avila. 1986. Phosphorylation of tubulin enhances its interaction with membranes. *Nature (Lond.)* 323:827-828.
13. Hataway, M. G., and J. A. Traugh. 1982. Casein kinases: multipotential protein kinases. *Curr. Top. Cell. Regul.* 21:101-127.
14. Herrmann, H., M. S. Dalton, and G. Wiche. 1985. Microheterogeneity of microtubule-associated proteins, MAP1 and MAP2 and differential phosphorylation of individual subcomponents. *J. Biol. Chem.* 260:5797-5803.
15. Kadowaki, T., Y. Fujita-Yamaguchi, E. Nishida, F. Takaku, T. Akiyama, S. Kathuria, Y. Akamura, and M. Kasuga. 1985. Phosphorylation of tubulin and microtubule-associated proteins by the purified insulin receptor kinase. *J. Biol. Chem.* 260:4016-4020.
16. Laemmli, U. K. 1980. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* 227:680-685.
17. Maccioni, R. B., L. Serrano, J. Avila, and J. R. Cann. 1986. Characterization and structural aspects of the enhanced assembly of tubulin after removal of its carboxy terminal domain. *Eur. J. Biochem.* 156:375-381.
18. Maggio, F., D. A. Deana, and L. A. Pinna. 1981. Endogenous phosphate acceptor proteins for rat liver cytosolic casein-kinases. *J. Biol. Chem.* 251:11958-11961.

19. Nestler, E. J., and P. Greengard. 1984. Protein phosphorylation in the nervous system. John Wiley & Sons, Inc., New York. 274-277.
20. Pfeffer, R. S., D. G. Drubin, and R. B. Kelly. 1983. Identification of three coated vesicle components as α - and β -tubulin linked to a phosphorylated 50,000-dalton polypeptide. *J. Biol. Chem.* 97:40-47.
21. Pinna, L. A., V. A. Deano, and F. Meggros. 1979. Structural features determining the site specificity of a rat liver cAMP independent protein kinase. *Biochem. Biophys. Res. Commun.* 87:114-120.
22. Ponsingl, H., E. Krauhs, M. Little, M. Kempf, R. Hofer-Warbineck, and W. Ade. 1982. Amino-acid sequence of α - and β -tubulins from pig brain: heterogeneity and regional similarity to muscle proteins. *Cold Spring Harbor Symp. Quant. Biol.* 46:191-198.
23. Rappaport, L., J. F. Leterrier, A. Virion, and J. Nunez. 1976. Phosphorylation of microtubule-associated proteins. *Eur. J. Biochem.* 62:539-549.
24. Rodnight, R., and B. E. Levin. 1964. Phosvitin kinase from brain: activation by ions and subcellular distribution. *Biochem. J.* 93:84-91.
25. Sandoval, I. V., and P. Cuatrecasas. 1976. Protein kinase associated with tubulin: affinity chromatography and properties. *Biochemistry.* 15:3424-3432.
26. Serrano, L., J. C. de la Torre, R. B. Maccioni, and J. Avila. 1984. Involvement of the carboxyl-terminal domain of tubulin in the regulation of its assembly. *Proc. Natl. Acad. Sci. USA.* 81:5989-5993.
27. Serrano, L., J. Avila, and R. B. Maccioni. 1984. Controlled proteolysis of tubulin by subtilisin: localization of the site for MAP₂ interaction. *Biochemistry.* 23:4675-4681.
28. Serrano, L., E. Montejó, M. A. Hernández, and J. Avila. 1985. Localization of the tubulin binding site for tau protein. *Eur. J. Biochem.* 153:595-600.
29. Shelanski, M. L., F. Gaskin, and C. R. Cantor. 1973. Microtubule assembly in the absence of added nucleotides. *Proc. Natl. Acad. Sci. USA.* 70:765-768.
30. Sloboda, R. D., S. A. Rudolph, J. L. Rosenbaum, and P. Greengard. 1975. Cyclic AMP-dependent endogenous phosphorylation of a microtubule-associated protein. *Proc. Natl. Acad. Sci. USA.* 72:177-181.
31. Sonderegger, P., R. Jausi, H. Gunning, K. Brunshweiler, and P. Christien. 1982. Peptide mapping of protein bands from polyacrylamide gel electrophoresis by chemical cleavage in gel pieces. *Anal. Biochem.* 122:298-301.
32. 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.
33. Sullivan, K. F., C. J. Havercroft, P. S. Machlin, and D. W. Cleveland. 1986. Sequence and expression of the chicken β 5 and β 4 tubulin genes define a pair of divergent β tubulin genes with complementary patterns of expression. *Mol. Cell. Biol.* 6:4409-4418.
34. Vallee, R. B., M. J. DiBartolomeis, and W. E. Theurkauf. 1981. A protein kinase associated with the projection portion of MAP2. *J. Cell Biol.* 90:568-576.
35. Wandosell, F., L. Serrano, M. A. Hernández, and J. Avila. 1986. Phosphorylation of tubulin by a calmodulin-dependent protein kinase. *J. Biol. Chem.* 261:10332-10339.
36. Deleted in proof.
37. Weisenberg, R. C. 1972. Microtubule formation in vitro in solutions containing low calcium concentrations. *Science (Wash. DC).* 177:1104-1105.
38. Yamamoto, H., K. Fukunaga, S. Goto, E. Tanaka, and E. Miyamoto. 1985. Ca²⁺ calmodulin-dependent regulation of microtubule formation via phosphorylation of microtubule-associated protein 2, tau-factor and tubulin, and comparison with the cyclic AMP-dependent phosphorylation. *J. Neurochem.* 44:759-767.
39. Yoshikawa, K., H. Usui, M. Imazu, H. Tsukamoto, and M. Takeda. 1985. Comparison of tyrosine protein kinase in membrane fraction from mouse liver and Ehrlich ascites tumor. *J. Biol. Chem.* 260:15091-15099.