Phosphorylation of β_{III}-Tubulin†

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ABSTRACT: There is considerable evidence that mammalian β-tubulin is phosphorylated. Specifically, of the seven β isotypes, the phosphorylated one is β_{III}, the isotype found almost entirely in neurons. The phosphate is added at a serine and perhaps a tyrosine near the C-terminus. All the evidence to date has been gathered by growth of cells and tissues in the presence of radioactive inorganic phosphate followed by tubulin isolation and determination of the labeled tubulin; thus, the actual extent of phosphorylation of β_{III} is unknown. Nor is it known if α-tubulin and the other β isotypes are phosphorylated by a mechanism which would not be revealed by previous experiments. In addition, the role of tubulin phosphorylation is unknown. We have purified the $\alpha\beta_{II}$-, $\alpha\beta_{III}$-, and $\alpha\beta_{IV}$-tubulin dimers from bovine brain and have determined their phosphate content chemically. We have found that α-tubulin is not phosphorylated and neither are the β_{II} or β_{IV} isotypes. However, β_{III} is phosphorylated with a stoichiometry of about 1.52 mol/mol. We have found that the phosphate on β_{III} is resistant to a wide variety of phosphatases except for human erythrocyte phosphatase 2A and that removal of the phosphate inhibits microtubule assembly *in vitro* stimulated by microtubule-associated protein 2 (MAP 2). However such an inhibition was not evident when microtubule assembly was induced in the absence of microtubule-associated proteins. Our results suggest the possibility that β_{III} phosphorylation may play a role in regulating microtubule assembly *in vivo*.

Microtubules are long cylindrical organelles playing critical roles in a variety of processes such as mitosis, axonal transport, and axonemal motility. The major constituent of microtubules is the 100 kDa protein tubulin, which is a heterodimer consisting of two 50 kDa polypeptide chains designated α and β (Ludueña et al., 1977; Dustin, 1984). Both α- and β-tubulin exist in several isotypic forms, encoded by different genes (Ludueña, 1993). In addition, the tubulin molecule is subject to several posttranslational modifications, including tyrosinolation and detyrosinolation of α (Raybin & Flavin, 1977; Thompson, 1982; Mullins et al., 1994), acetylation of α (LeDizet & Piperno, 1987), glutamylation of α and β (Eddé et al., 1990; Alexander et al., 1991; Redeker et al., 1992; Rüdiger et al., 1992; Mary et al., 1994), and phosphorylation of the β_{III} isotype (Ludueña et al., 1988; Diaz-Nido et al., 1990). It is likely that these modifications are of great significance in modulating the assembly or properties of microtubules, but their precise roles have not been determined.

The first of these modifications to be observed *in situ* was the phosphorylation of β-tubulin, reported in 1972 by Eipper (1972), who incubated rat brain slices with radioactive $^{32}PO_4^{3-}$ and found that tubulin purified therefrom was labeled in the β subunit. Similar results were found by others with reported stoichiometries[1] ranging from 0.28 to 0.34 mol of phosphate/mol of tubulin (Reddington & Lagnado, 1973; Piras & Piras, 1974). Eipper (1974) showed that the labeled phosphate was attached to a serine in the C-terminal region of β-tubulin and that the stoichiometry of labeling was 0.7 ± 0.1 mol/mol. More recently, Gard and Kirschner (1985) showed that when neuroblastoma cells were induced to differentiate in the presence of $^{32}PO_4^{3-}$, only one specific isotype of β incorporated the labeled phosphate. Later, Ludueña et al. (1988) found that the labeled isotype was β_{III} [using the nomenclature of Sullivan and Cleveland (1986)] and that the labeled phosphate was incorporated near the C-terminus. In an analogous approach, Diaz-Nido et al. (1990) injected adult rats with $^{32}PO_4^{3-}$, purified the tubulin, and located the labeled phosphate on Ser^{444} of the β_{III} isotype. A limitation of these approaches is that it is possible that the extent of phosphorylation is underestimated due to the fact that a putative phosphorylation site on the tubulin molecule may already be phosphorylated and would therefore not acquire labeled phosphate during incubation with $^{32}PO_4^{3-}$. By the same argument, it is conceivable that α or the other β isotypes are phosphorylated but that their state of phosphorylation does not change upon differentiation of neuroblastoma cells. Also, neuroblastoma cells are transformed, and the results observed with them may not always reflect the situation in a normal adult mammalian brain neuron.

In another approach to measuring the extent of phosphorylation, useful when substantial quantities of tubulin are available, the amount of phosphate attached to the tubulin molecule can be determined chemically. This has the advantage of being able to detect all the protein-bound phosphate, not merely that which is incorporated during the incubation. This approach was used by Bhattacharyya and

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[1] Stoichiometries of phosphorylation of tubulin reported in the early literature have been recalculated, assuming a molecular mass of 100 kDa for the tubulin dimer. In earlier work, molecular mass values of tubulin ranging from 100 kDa to 116 kDa were reported.

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Wolff (1974), who found that bovine thyroid tubulin contained 0.71 mol of phosphate/mol of tubulin, by Lagnado and Tan (1975), using rat, pig, and guinea pig brain, who reported 0.31−0.73 mol/mol, and by Kirschner et al. (1975), who reported 0.41 mol of phosphate/mol of pig brain tubulin. Although this work established that normal adult brain tubulin is phosphorylated, it did not define which isotype of tubulin is phosphorylated or even whether the phosphate is on the α or β subunit. The role of tubulin phosphorylation is uncertain. Attempts to remove the phosphate from native tubulin have been unsuccessful (Yamamoto et al., 1988).

Both α- and β-tubulin exist as numerous isotypes, differing in their amino acid sequences (Sullivan, 1988). The β isotypes differ among themselves more than do the α isotypes (Little & Seehaus, 1988). The isotypic composition of bovine brain β-tubulin is as follows: β_I, 3%; β_{II}, 58%; β_{III}, 25%; and β_{IV}, 13% (Banerjee et al., 1988). We have developed monoclonal antibodies specific for three β-tubulin isotypes, namely, β_{II}, β_{III}, and β_{IV} (Banerjee et al., 1988, 1990, 1992). We have used these antibodies to prepare isotypically purified tubulin dimers, designated as $\alpha\beta_{II}$, $\alpha\beta_{III}$, and $\alpha\beta_{IV}$. We have separated α and β from each of these dimers and have done inorganic phosphate determinations on the α and β chains from each dimer. We have found that of the three β isotypes, only β_{III} is phosphorylated. The partial dephosphorylation of $\alpha\beta_{III}$-tubulin by human erythrocyte phosphatase 2A led to approximately 32% loss in its ability to polymerize into microtubules in the presence of microtubule-associated protein 2 (MAP 2). The microtubules obtained from the dephosphorylated tubulin were morphologically indistinguishable from the normal microtubules. In contrast, dephosphorylation of β_{III} did not affect its ability to polymerize in the absence of microtubule-associated proteins (MAPs). Our results raise the possibility that phosphorylation of β_{III} may play a role in the regulation of microtubule assembly *in vivo*, perhaps targeting the tubulin−MAP interaction.

These results have been published in preliminary form elsewhere (Khan & Ludueña, 1990, 1991a, 1992).

MATERIALS AND METHODS

Materials. HCl Ultrex Ultrapure was from J. T. Baker (Phillipsburg, NJ). The phosphatase assay system was purchased from Gibco BRL (Gaithersburg, MD). Alkaline phosphatases (types IX and III) (EC 3.1.3.1) from beef liver and potato and acid phosphatases from human prostate and bovine semen were from Sigma Chemical Co. (St. Louis, MO). Calf intestine alkaline phosphatase was from Boehringer Mannheim, GmbH (Indianapolis, IN). Lambda phosphatase was obtained from New England Biolabs (Beverly, MA). Human erythrocyte phosphatase 2A was obtained from Upstate Biotechnology Inc., Lake Placid, NY. Calyculin A was obtained from Moana Bioproducts (Honolulu, Hawai). Goat anti-mouse IgG conjugated to horseradish peroxidase and peroxidase substrate kit were from BioRad (Richmond, CA).

Preparation of Immunoaffinity Columns. The monoclonal antibodies specific for the β_{II}-, β_{III}-, and β_{IV}-tubulin isotypes were prepared and isolated from cell-free supernatants as previously described (Banerjee et al., 1988; 1990; 1992; Roach et al., 1988). Approximately 305, 340, and 270 mg of purified IgG specific, respectively, for β_{II}, β_{III}, and β_{IV} were coupled separately to CNBr-activated Sepharose-4B as instructed by Pharmacia. These immunoaffinity columns will hereafter be designated as anti-β_{II}, anti-β_{III}, and anti-β_{IV} columns.

Purification of Tubulin Isotypes. Microtubules were prepared from bovine cerebra and tubulin was purified from the microtubules by phosphocellulose chromatography following the procedure of Fellous et al. (1977). The isotypically purified tubulin dimers were prepared as follows. For $\alpha\beta_{II}$-tubulin, 70 mg of phosphocellulose-purified tubulin in 27 mL of 2-(*N*-morpholino)ethanesulfonic acid (Mes)[2] buffer (0.1 M Mes, pH 6.4, 0.5 mM $MgCl_2$, 1 mM EGTA, 0.1 mM EDTA, and 1 mM GTP) was loaded on the anti-β_{III} column (2.5 × 14.5 cm) previously equilibrated with the same buffer at 10 °C. After 1 h, the column was washed with 200 mL of Mes buffer at a flow rate of ∼2 mL/min, and the unbound fractions (2.5 mL each) containing a mixture of unbound $\alpha\beta_{II}$ and $\alpha\beta_{IV}$ were pooled and concentrated on an Amicon XM50 Diaflo Ultrafilter. The concentrate (∼40 mg of $\alpha\beta_{II}$ + $\alpha\beta_{IV}$ in 25 mL) was loaded on an anti-β_{IV} column (2.5 × 13.5 cm) in Mes buffer at 10 °C, and after 30 min the column was washed with 200 mL of Mes buffer, and the unbound fractions containing the $\alpha\beta_{II}$ dimers were concentrated and frozen in liquid nitrogen. Protein was quantitated by the method of Lowry et al. (1951). For $\alpha\beta_{III}$-tubulin, 60 mg of phosphocellulose-purified tubulin was passed through the anti-β_{II} column (2.5 × 15 cm), and the unbound fractions containing $\alpha\beta_{III}$ and $\alpha\beta_{IV}$ were pooled, concentrated, and loaded onto the anti-β_{IV} column. The unbound fractions from this column contained electrophoretically pure $\alpha\beta_{III}$-tubulin. These fractions were concentrated and frozen in liquid nitrogen. All other experimental conditions were the same as in the purification of $\alpha\beta_{II}$. For $\alpha\beta_{IV}$-tubulin, 60 mg of phosphocellulose-purified tubulin was loaded on the anti-β_{II} column, and the unbound fractions containing a mixture of $\alpha\beta_{III}$ and $\alpha\beta_{IV}$ were pooled, concentrated, and passed through the anti-β_{III} column. The unbound fractions containing pure $\alpha\beta_{IV}$ were concentrated and frozen in liquid nitrogen. All isotypically purified tubulins were stored at −80 °C until ready for use.

The relative amounts of $\alpha\beta_{III}$ in each tubulin sample were measured by subjecting the tubulin to SDS−PAGE on 5.5% gels (Laemmli, 1970). Tubulin samples were reduced and carboxymethylated prior to SDS−PAGE (Crestfield et al., 1963). Under these conditions the β_{III} isotype has an electrophoretic mobility distinctly different from those of either the β_{II} or the β_{IV} isotypes, which comigrate (Ludueña et al., 1982; Banerjee et al., 1990).

Isolation of Tubulin Subunits. α and β subunits were prepared from isotypically purified dimers according to the following procedure. First, 2.6 mg of the reduced and carboxymethylated dimer was subjected to SDS−PAGE (Laemmli, 1970) on a 5.5% gel (0.15 × 14 × 14 cm). The α and β subunits of tubulin were visualized using a variation of the method of Nelles and Bamburg (1976) in which the gel was soaked in a copious volume of 4 M KCl, instead of 1 M KCl. The bands were excised and cut into ∼1.6 × 2.0 mm pieces, and the protein entrapped in the gel was

[2] Abbreviations: EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; GTP, guanosine 5′-triphosphate; Mes, 2-(*N*-morpholino)ethanesulfonic acid; PBS, phosphate-buffered saline (0.01 M sodium phosphate, pH 7.0, 0.15 M NaCl, and 0.01 M EDTA); PCT, phosphocellulose-purified isotypically unfractionated tubulin; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

electroeluted using Centricon-30 and Centriluter (Amicon, Danvers, MA). The electroelution was carried out at 40 mA overnight at room temperature in 0.025 M Tris, 0.192 M glycine, pH 8.3. The protein was recovered from Centricon as described by the manufacturer and stored in Mes buffer (without GTP) at −80 °C until used.

Western Blots. Immunoblotting of gels was carried out as previously described (Banerjee et al., 1990) except that the second antibody, instead of being radioactive, was conjugated with horseradish peroxidase (1:1000 dilution in 25 mM Tris-HCl, pH 7.0, 0.12 M NaCl, 0.2% Tween-20, and 0.1% thimerosal).

Determination of P_i. Phosphorylation of protein samples was measured by the procedure of Buss and Stull (1983) in which the sample is ashed and the phosphate content is determined. A control tube without any protein was also run parallel to the test samples to detect any background contamination.

Inhibition of Protein Phosphatase Activities of Human Erythrocyte and E. coli Lambda Phosphatases by Calyculin A and GTP. The phosphatase substrate, ^{32}P-labeled phosphorylase *a*, was prepared from a phosphatase assay system kit (Gibco BRL) as described by the manufacturer. The phosphatase (1.5−2.0 IU) and ^{32}P-labeled phosphorylase *a* (90 μg) were mixed in the presence or absence of either calyculin A (0−6 μM) or GTP (0−1 mM) in 0.1 M Mes, 0.1 mM EDTA, 1 mM EGTA, 0.5 mM $MgCl_2$, and 1 mM β-ME, pH 6.4 (plus 2 mM $MnCl_2$ in the case of lambda phosphatase). The mixture was incubated at 30 °C for 10 min, and ^{32}P liberated by the phosphatase was measured in Beckman LS-7000 scintillation counter.

Dephosphorylation of Tubulin. (A) By Acid and Alkaline Phosphatases. Aliquots (0.44−1.25 mg) of isotypically pure tubulin were mixed with 5−15 units of alkaline phosphatase in 0.1 M Mes buffer containing 2 mM EDTA and 2 mM EGTA, pH 6.4 or 6.8, as indicated, or with 30−85 μg of acid phosphatase in 0.1 M sodium citrate buffer, pH 4.8. The samples were incubated at 37 °C for 30−90 min.

(B) By Human Erythrocyte and E. coli Lambda Phosphatases. The dephosphorylation buffer contained 0.1 M Mes, 0.1 mM EDTA, 1 mM EGTA, 0.5 mM $MgCl_2$, and 1 mM β-mercaptoethanol, pH 6.4. In the case of lambda phosphatase, the buffer also contained 2 mM $MnCl_2$. Both lambda and erythrocyte phosphatases were equilibrated with the respective dephosphorylation buffers prior to their use; 1.6 IU of erythrocyte phosphatase or 400 units of lambda phosphatase was incubated with 1.34−1.6 mg of $\alpha\beta_{III}$-tubulin at 30 °C for 20 min followed by the addition of another aliquot of the phosphatase, and the incubation was continued for another 30 min. The dephosphorylation was terminated with 5 μM calyculin A and 1 mM GTP. The protein was precipitated with 20% TCA, and its phosphate content was measured as described above.

Tubulin Polymerization. Phosphatase-treated tubulin and untreated tubulin in Mes buffer, pH 6.4, were mixed separately with Mes buffer containing 1 mM GTP and either MAP 2 or 4 M glycerol and 6 mM $MgCl_2$ at 0 °C. The temperature of the samples was raised from 4 to 37 °C, and tubulin polymerization was followed by either of two methods.

(A) Sedimentation. Aliquots (60 μL) were withdrawn at various time intervals and centrifuged for 4 min in the Beckman airfuge at 175000*g* (Khan & Ludueña, 1991b). The polymer concentrations in the pellets were measured by first solubilizing the pellet in a final volume of 100 μL of 0.05 N NaOH and then quantitating the total protein in the sample (Lowry et al., 1951).

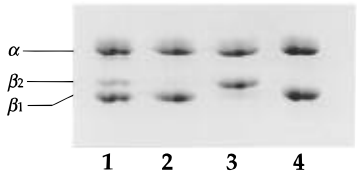

FIGURE 1: Analysis of isotypically purified tubulin dimers by polyacrylamide gel electrophoresis. Reduced and carboxymethylated samples (6.3 μg) were subjected to electrophoresis on 5.5% polyacrylamide gels as described in the text. Samples were as follows: 1, phosphocellulose-purified tubulin; 2, $\alpha\beta_{II}$; 3, $\alpha\beta_{III}$; 4, $\alpha\beta_{IV}$. The gels were stained with Coomassie brilliant blue. Of the two β-tubulin bands seen in unfractionated tubulin, β_1 contains the β_I, β_{II}, and β_{IV} isotypes, and β_2 contains the $\alpha\beta_{III}$ isotype (Sullivan, 1988).

(B) Turbidimetry. The polymerization of tubulin was followed by measuring the change in turbidity at 350 nm on a Gilford-250 spectrophotometer equipped with a chart recorder (Gaskin et al., 1974). The rates of polymerization were computed from the maximum slopes of the polymerization curves and expressed as the change in absorbance per minute.

Tubulin:MAP2 Ratio. $\alpha\beta_{III}$-Tubulin was incubated with erythrocyte phosphatase, and the reaction was terminated with 6 μM calyculin A. In the control sample, the phosphatase was incubated with calyculin A before the addition of $\alpha\beta_{III}$-tubulin. The assembly of $\alpha\beta_{III}$-tubulin in the presence of MAP 2 was followed by sedimentation. The pellet was solubilized in 0.05 N NaOH, mixed with sample buffer, and electrophoresed on a 6.5% polyacrylamide gel (Laemmli, 1970). The protein bands in the gel were stained with Coomassie Brilliant blue R and scanned on a Macintosh IIci computer using the public domain NIH Image program (Anonymous FTP from zippy.nimh.nih.gov). The integrated peak areas of tubulin and MAP 2 were compared to give the tubulin:MAP 2 ratio.

Electron Microscopy. The samples for electron microscopy were prepared as described earlier (Khan & Ludueña, 1991b).

RESULTS

Tubulin isotypes were purified as described under Materials and Methods. Thus, $\alpha\beta_{II}$ was purified by passing through anti-β_{III} and anti-β_{IV} columns; $\alpha\beta_{III}$ on anti-β_{II} and anti-β_{IV} columns; and $\alpha\beta_{IV}$ on anti-β_{II} and anti-β_{III} columns. Since the anti-β_{II} can also bind $\alpha\beta_I$ (Banerjee et al., 1988), it is assumed that the purified $\alpha\beta_{III}$ and $\alpha\beta_{IV}$ are almost free of $\alpha\beta_I$. However, based on the known isotype composition of unfractionated bovine brain tubulin (Banerjee et al., 1988), one can calculate that the purified $\alpha\beta_{III}$ has a 5% contamination with $\alpha\beta_I$. The resulting purified dimers are shown in Figure 1. The band in the $\alpha\beta_{III}$ sample with the mobility of β_{II} and β_{IV} was identified as β_{II} by immunoblotting. Quantitation of the immunoblots suggested that the isotypic composition of the β-tubulin of the samples was as follows: $\alpha\beta_{II}$ (5% β_I, 95% β_{II}), $\alpha\beta_{III}$ (1% β_I, 13% β_{II}, 86% β_{III}), $\alpha\beta_{IV}$ (100% β_{IV}).

The phosphorylation of the purified isotypes was determined and compared to that of unfractionated tubulin. Averaging the results of duplicate experiments, unfractionated tubulin had a phosphate content of 0.36 mol/mol of

Table 1: Phosphate Content of Subunits of Purified Isotypes[a]

dimer	subunit	phosphate content (mol of P_i/50 kDa)
PCT	α	0.08, 0.00
	β	0.34, 0.26
$\alpha\beta_{II}$	α	0.00, 0.03
	β	0.04, 0.04
$\alpha\beta_{III}$	α	0.10, 0.08
	β	1.41, 1.43
$\alpha\beta_{IV}$	α	0.05, 0.02
	β	0.01, 0.00

[a] Subunits of isotypically pure tubulins were prepared as described in the text. Approximately 400 μg of each of the subunits was processed for phosphate determination as described in the text.

Table 2: Effect of Phosphatase Treatment on the Phosphate Content of PCT and $\alpha\beta_{III}$-Tubulin Dimers[a]

treatment	phosphate content (mol of P_i/100 kDa) PCT[b]	$\alpha\beta_{III}$
none	0.39 ± 0.10[c]	1.45 ± 0.16[d]
acid phosphatase		
human prostatic	0.32	1.40
bovine semen	0.36	nd[e]
sweet potato	nd	1.56
potato	0.40	1.38
alkaline phosphatase		
calf intestine	0.46	1.49
bovine liver	nd	1.64
potato	0.32	1.56

[a] Samples of tubulin were digested with the indicated phosphatases, and the phosphate contents of the samples were determined as described in the text. Specific digestion conditions were as follows: for human prostatic acid phosphatase, 0.671 mg of PC-T, 0.940 mg of $\alpha\beta_{III}$, and 30 μg of phosphatase; for bovine semen acid phosphatase, 0.540 mg of PC-T and, 40 μg of phosphatase; for sweet potato acid phosphatase, 0.488 mg of $\alpha\beta_{III}$, and 30 μg of phosphatase; for potato acid phosphatase, 0.494 mg of PC-T, 0.543 mg of $\alpha\beta_{III}$, and 40 μg of phosphatase; for calf intestine alkaline phosphatase, 0.494 mg of PC-T, 0.293 mg of $\alpha\beta_{III}$, and 8.6 units of phosphatase in 0.1 M Pipes, pH 6.9, 2 mM EDTA, 2 mM EGTA; for bovine liver alkaline phosphatase, 0.748 mg of $\alpha\beta_{III}$ and 10.6 units of phosphatase; for potato alkaline phosphatase, 0.820 mg of PC-tubulin, 0.748 mg of $\alpha\beta_{III}$, and 15.0 units phosphatase. [b] Unfractionated tubulin (phosphocellulose-purified tubulin). [c] Represents an average of 7 samples. [d] Represents an average of 5 samples. [e] nd = not determined.

tubulin, while $\alpha\beta_{II}$, $\alpha\beta_{III}$, and $\alpha\beta_{IV}$ had contents of 0.05, 1.52, and 0.01 mol/mol of tubulin, respectively. Apparently, high phosphorylation of $\alpha\beta_{III}$ was confirmed when the α and β subunits were separated and their phosphate contents were determined (Table 1). As can be seen, the greatest phosphorylation was seen in β_{III}, about 1.4 moles of phosphate/mol of tubulin. A very small amount of phosphorylation was seen with the α subunit from the $\alpha\beta_{III}$ dimer, which on average contained about 0.09 mol of phosphate per subunit. The sum of these two numbers should give the total phosphorylation of the dimer, which would be 1.49, very close to the value obtained by phosphate determination on the whole $\alpha\beta_{III}$ dimer.

We also examined the effect of a number of acid and alkaline phosphatases on the phosphate content of isotypically purified $\alpha\beta_{III}$ dimers. Table 2 shows that neither unfractionated tubulin nor $\alpha\beta_{III}$ could be dephosphorylated by most of these phosphatases. However, we have found that human erythrocyte phosphatase 2A and *E. coli* lambda phosphatase were able to significantly dephosphorylate $\alpha\beta_{III}$-tubulin (Table 3). Human erythrocyte phosphatase removed

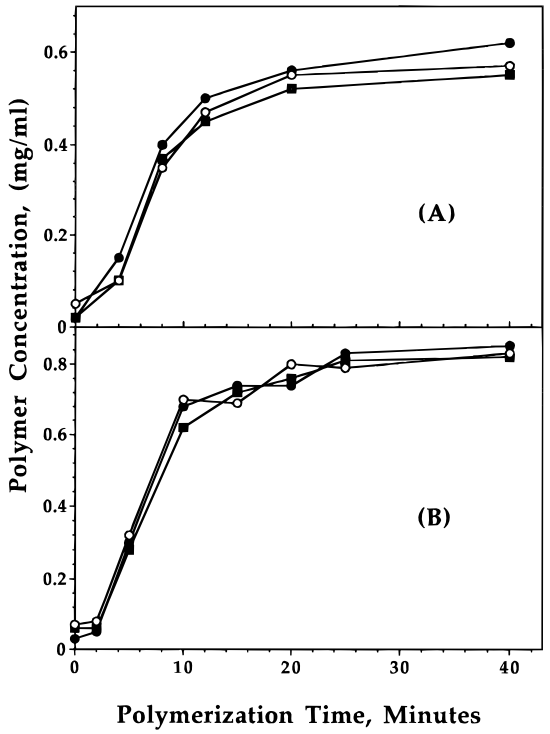

FIGURE 2: Assembly of phosphatase-treated unfractionated tubulin and $\alpha\beta_{III}$-tubulin in the presence of glycerol and $MgCl_2$. Unfractionated tubulin (A) or $\alpha\beta_{III}$-tubulin (B) was dephosphorylated with human erythrocyte phosphatase 2A, and the sample was polymerized at 37 °C in the presence of 4 M glycerol, 6 mM $MgCl_2$, and 1 mM GTP as described under Materials and Methods. The assembly was followed by sedimentation, and the polymer concentrations in the samples were calculated as described in the text. The final concentrations of unfractionated and $\alpha\beta_{III}$-tubulin were 1.4 and 1.1 mg/mL, respectively. Tubulin was treated with phosphatase and calyculin A in a specific order as given below: (○) untreated tubulin; (●) phosphatase + tubulin + calyculin A; and (■) phosphatase + calyculin A + tubulin.

64% of the phosphate from $\alpha\beta_{III}$. On the other hand, the lambda phosphatase removed only 15% of the phosphate.

The dephosphorylation of both unfractionated and $\alpha\beta_{III}$-tubulin did not alter either the pattern or the extent of their polymerization when the assembly was induced by 4 M glycerol−6 mM $MgCl_2$ (Figure 2). In contrast to the above finding, the removal of the phosphate had a significant inhibitory effect on microtubule assembly of $\alpha\beta_{III}$ in the presence of MAP 2 (Table 3 and Figure 3). The sample of $\alpha\beta_{III}$ that had been digested with human erythrocyte phosphatase 2A assembled much more slowly than did the untreated control. The rate of assembly was inhibited by 35% and the extent of assembly at 1 h by 32% as measured by sedimentation (Table 3, Figure 3).

Calyculin A is a potent inhibitor of erythrocyte phosphatase 2A and phosphatase 2A isolated from other sources (Suganuma et al., 1992; Quinn et al., 1993). Nanomolar quantities of calyculin A are known to inactivate phosphatases 2A (Suganuma et al., 1992; Quinn et al., 1993). However, by using ^{32}P-labeled phosphorylase *a*, we found that erythrocyte phosphatase 2A could be completely inactivated by 5 μM calyculin A (data not given). GTP, although unrelated to calyculin A, has been shown to inhibit the activity of phosphatase *S* isolated from canine cardiac muscle (Hsiao et al., 1978); however, its use as an inhibitor of erythrocyte phosphatase 2A and lambda phosphatase has not been reported earlier. Under our buffer conditions, namely,

Table 3: Effect of Human Erythrocyte Phosphatase 2A and *E. coli* Lambda Phosphatase on the Phosphate Content and the Assembly of $\alpha\beta_{III}$-Tubulin

treatment[a]	P_i content of $\alpha\beta_{III}$ (mol/mol)[b]	MT assembly (% of control)[c]	rate constant (k = OD/min)[e]
(1) $\alpha\beta_{III}$ (no treatment)	1.44	100	nd
(2) erythrocyte phosphatase + calyculin A + $\alpha\beta_{III}$-tubulin	1.55	105	8.6×10^{-2}
(3) erythrocyte phosphatase + $\alpha\beta_{III}$-tubulin + calyculin A	0.56	73	5.6×10^{-2}
(4) λ phosphatase + calyculin A & GTP + $\alpha\beta_{III}$	1.51	100[d]	nd
(5) λ phosphatase + $\alpha\beta_{III}$ + calyculin A & GTP	1.29	90	nd

[a] The $\alpha\beta_{III}$-tubulin was incubated with erythrocyte phosphatase, and the reaction was terminated with 6 μM calyculin A as described in the text. In the control sample, the phosphatase was incubated with calyculin A before the addition of $\alpha\beta_{III}$-tubulin. In the case of lambda (λ) phosphatase, the reaction was terminated with 6 μM calyculin A and 1 mM GTP. [b] The phosphate determination was carried out on an aliquot before the addition of MAP 2. [c] The assembly of $\alpha\beta_{III}$-tubulin in the presence of MAP 2 was followed by sedimentation as described in the legend to Figure 3. [d] The assembly was followed by sedimentation (data not shown) as described in Materials and Methods. [e] The rate constants were determined by following the assembly turbidimetrically as described in Materials and Methods. nd = not determined.

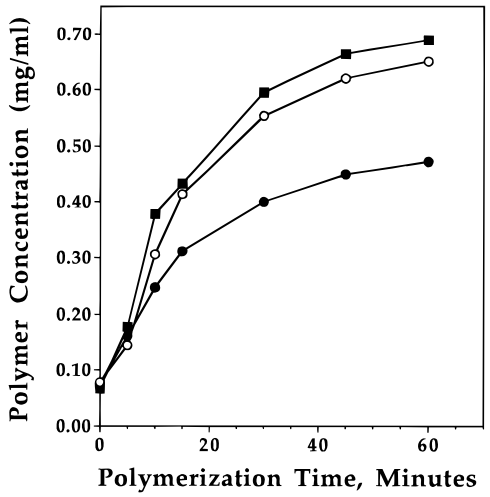

FIGURE 3: Assembly of phosphatase-treated $\alpha\beta_{III}$-tubulin in the presence of MAP 2. $\alpha\beta_{III}$-Tubulin was dephosphorylated with human erythrocyte phosphatase 2A, and the sample was polymerized at 37 °C in the presence of MAP 2 and 1 mM GTP as described under Materials and Methods. The assembly was followed by sedimentation, and the polymer concentrations in the samples were calculated as described in the text. The final concentrations of tubulin and MAP 2 were 1.33 and 0.3 mg/mL, respectively. Tubulin was treated with phosphatase and calyculin A in a specific order as given below: (○) untreated $\alpha\beta_{III}$-tubulin; (●) phosphatase + $\alpha\beta_{III}$-tubulin + calyculin A; and (■) phosphatase + calyculin A + $\alpha\beta_{III}$-tubulin.

0.1 M Mes, 0.1 mM EDTA, 1 mM EGTA, 0.5 mM $MgCl_2$, and 1 mM β-mercaptoethanol, pH 6.4 (plus 0.2 mM $MnCl_2$ in the case of lambda phosphatase), the dephosphorylation of ^{32}P-labeled phosphorylase *a* by either erythrocyte or lambda phosphatases could be completely inactivated by 1 mM GTP (data not shown here). Therefore, 6 μM calyculin A and 1 mM GTP were routinely added to the samples after phosphatase incubation in order to prevent dephosphorylation of MAP 2, which is known to stimulate assembly (Yamamoto et al., 1988). In order to address the remote possibility that the phosphatase might be contaminated with some other enzyme, such as a protease, which, by degrading MAP 2, could have deleterious effects on microtubule assembly, calyculin A preincubation was used as a control. We previously determined the concentration of calyculin A which would completely inhibit the human erythrocyte phosphatase 2A at the concentrations of the latter used in our experiments. When the control sample was preincubated with calyculin A prior to phosphatase treatment, assembly was not inhibited and no dephosphorylation occurred (Figures 2 and 3). Thus, the inhibitory effect on assembly when tubulin was incubated with phosphatase prior to calyculin A addition was due to the phosphatase activity rather than another contaminating enzyme.

In evaluating the above experiments in which the effect of dephosphorylation on microtubule polymerization is examined, it is important to investigate the possibility that the phosphatase, either by its presence alone or else by its activity, could induce a nonspecific aggregation of tubulin that might be masked in the presence of assembly-inducing factors such as MAP 2 or the glycerol−Mg buffer. Such an aggregate might even interfere with the microtubule assembly induced by these factors. When using purified isotypes, whose full range of heterologous protein−protein interactions have not yet been explored, it is particularly important to consider this possibility. In order to accomplish this, we performed control experiments in which either unfractionated tubulin or $\alpha\beta_{III}$ was incubated in the absence of the assembly-promoting factors, both with and without erythrocyte phosphatase treatment and also with phosphatase present but inhibited by calyculin A. In these experiments, samples were taken for measurement of aggregation at various time points between 0 and 60 min (data not shown). In one experiment, tubulin was incubated in the same conditions as in Figure 2A, except that glycerol was absent from the buffer and the $MgCl_2$ concentration was 0.5 mM. Under these conditions, unfractionated tubulin showed no significant assembly: the concentration of pelletable protein after 40 min was 0.059 mg/mL, an increase of 0.030 mg/mL over the concentration of 0.029 mg/mL at 0 min. In the presence of active phosphatase, the increase was 0.013 mg/mL, and in the presence of phosphatase inhibited by calyculin A, it was 0.050 mg/mL. Compared to the extent of assembly shown in Figure 2A, the increases seen for tubulin alone, for tubulin with active phosphatase, and for tubulin with inactive phosphatase were, respectively, 6%, 2%, and 2% of those obtained in the experiment shown in Figure 2A in the presence of glycerol−Mg buffer. In other words, without glycerol, there was little or no tubulin polymerization and the phosphatase made very little difference. In an experiment analogous to that shown in Figure 2B, using $\alpha\beta_{III}$ instead of unfractionated tubulin, but still in the absence of glycerol, there was again little or no aggregation and the phosphatase had no effect; the increases seen after 40 min for $\alpha\beta_{III}$ alone, for $\alpha\beta_{III}$ with active phosphatase, and for $\alpha\beta_{III}$ with inactive phosphatase were, respectively, 5%, 3%, and 0.3% of those seen in the experiment shown in Figure 2B in the presence of glycerol−Mg buffer. Finally, in an experiment identical to the one shown in Figure 3, using $\alpha\beta_{III}$, but in the absence of MAP 2, again there was no significant aggregation nor did the

phosphatase make any difference; the increases seen after 60 min for $\alpha\beta_{III}$ alone, for $\alpha\beta_{III}$ with active phosphatase and for $\alpha\beta_{III}$ with inactive phosphatase were, respectively, 2%, 0%, and 0% of those seen in the experiment shown in Figure 2B in the presence of MAP 2. In short, the tubulin showed little or no aggregation in the absence of glycerol and MAP2, nor did the phosphatase, either by its presence or by its activity, induce any aggregation.

The microtubules that were polymerized in the absence of MAPs from either unfractionated tubulin or $\alpha\beta_{III}$ after phosphatase treatment, as in Figure 2, were examined by electron microscopy and found to be morphologically indistinguishable from untreated microtubules. Similarly, the microtubules that were polymerized from $\alpha\beta_{III}$ in the presence of MAP 2 after treatment with erythrocyte phosphatase in the same conditions, as in Figure 3, were also examined by electron microscopy and found to be indistinguishable from untreated microtubules. These microtubules were also collected by sedimentation and analyzed by polyacrylamide gel electrophoresis (Laemmli, 1970); the ratio of MAP 2 to tubulin in microtubules from phosphatase-treated samples was not detectably different from that in microtubules from the untreated samples.

DISCUSSION

The present work clearly demonstrates that the β_{III} isotype of bovine brain tubulin is phosphorylated. It is likely, but not certain, that the phosphorylation occurs at Ser^{444} as was reported by Diaz-Nido et al. (1990). Since our phosphate determinations on the β_{III} subunit or $\alpha\beta_{III}$ dimer always yielded a stoichiometry in the range of 1.4−1.6 mol/mol, it is clear that Ser^{444} cannot be the only phosphorylated residue. Although bovine brain β_{III} has never been fully sequenced (Sullivan & Cleveland, 1986), it has been demonstrated that either Tyr^{437} or Ser^{444} or both may be phosphorylated (Alexander et al., 1991). It is possible, therefore, that some of the phosphate may be located on Tyr^{437}. The fact that these residues are not present in either β_{II} or β_{IV} may explain why neither β_{II} nor β_{IV} appears to be phosphorylated. Using the same reasoning, β_{III} has a serine, at position 239, which is not present in β_{II} or β_{IV} (Sullivan & Cleveland, 1986); conceivably, some of the phosphorylation of β_{III} is accounted for by phosphorylation of this serine. In adult bovine brain, $\alpha\beta_{III}$-tubulin makes up 25% of the total tubulin (Banerjee et al., 1988; 1992). Considering exclusive phosphorylation of only $\alpha\beta_{III}$-tubulin dimers in our tubulin preparations, one would expect unfractionated tubulin to be phosphorylated to an extent of 25% of that of $\alpha\beta_{III}$-tubulin, i.e., ~0.38 mol/mol. In fact, the observed phosphate stoichiometry of unfractionated tubulin (0.35) is very close to the expected number. This would suggest that even if the phosphorylation of tubulin occurs only in a very specific and relatively small amount of the tubulin, this phosphorylated species could be isolated in fully functional form with an approximately 100% yield.

The results in Table 1 show phosphorylation of $\alpha\beta_{II}$- and $\alpha\beta_{IV}$-tubulin, each averaging about 0.01−0.05 mol/mol, respectively. Similarly, phosphorylation of the α subunit of the β_{III}-tubulin dimer ranged from 0.08 to 0.10 with an average of 0.09 mol/mol of subunit. The question whether these extremely low stoichiometries represent a minor but specific phosphorylation or some environmental contamination is not absolutely clear. Since phosphate determinations on bovine serum albumin, a nonphosphorylated protein (Bylund & Krebs, 1975; Weller, 1979), yield stoichiometries ranging from 0 to 0.07 mol/mol, we assumed that any phosphorylation with a stoichiometry in this range is due to some unavoidable contamination. By this criterion, neither the $\alpha\beta_{II}$ nor the $\alpha\beta_{IV}$ isotypes seem to be phosphorylated whereas the phosphorylation of the β subunit of the $\alpha\beta_{III}$ dimer is certainly not artifactual.

The observed phosphorylation of the α subunit of the $\alpha\beta_{III}$ dimer is just marginally above background. Phosphorylation of α-tubulin has not previously been reported in mammalian tubulin; no phosphorylation of α was seen when radioactive phosphate was added to differentiating neuroblastoma cells (Ludueña et al., 1988; Gard & Kirshner, 1985). The most probable explanation for the apparent very low phosphorylation of the α subunit from the $\alpha\beta_{III}$ may simply be a trace amount of contamination, by the β subunit, of the purified α subunit. It is worth recalling, however, that although there is as yet no evidence for α phosphorylation in mammals, α-tubulin is phosphorylated in axonemal tubulin from sea urchin sperm and *Chlamydomonas* flagella (Stephens, 1975; Piperno & Luck, 1976). It would not be completely unexpected, therefore, if a fraction of mammalian α-tubulin were phosphorylated.

The phosphate on β_{III} was completely resistant to a variety of phosphatases, but could be partially removed by digestion with lambda phosphatase and even more so by the human erythrocyte phosphatase 2A. It is not absolutely clear why the phosphate of β_{III} should be so resistant to phosphatase digestion. One possibility, however, is suggested by its probable position in the amino acid sequence. As mentioned above, the most likely phosphorylated residues in β_{III} are Ser^{444} and, perhaps, Tyr^{437}. It is known that Glu^{438} in β_{III} is modified by addition of one to six glutamate residues to its γ-carboxyl group (Alexander et al., 1991). It is quite likely that the presence of these negatively charged glutamates in the vicinity of Ser^{444} and Tyr^{437} creates a unique environment which impedes access of the phosphatase to the phosphorylated residue. It is also conceivable that the partial removal of the phosphate by the human erythrocyte phosphatase 2A may reflect that the β_{III}-tubulin is a mixture of species glutamylated to different extents, with that which has six glutamates being perhaps completely resistant to the phosphatase and that which has no glutamates being completely susceptible, with intermediate forms being partly resistant.

The fact that most of the phosphatases (except those noted below) were unable to remove significant amounts of the phosphate attached to the $\alpha\beta_{III}$ dimer appears to contradict experiments in the literature in which the phosphate on the β_{III} isotype was removed by phosphatases (Serrano et al., 1987). In reality, there is little contradiction, since Serrano et al. (1987) were not measuring the intrinsic phosphate content of native tubulin before and after its treatment with phosphatase. Rather, they measured the additional phosphorylation of native tubulin by casein kinase because they observed that after digestion of tubulin with alkaline phosphatase, incubation with casein kinase II and [γ-^{32}P]ATP permits incorporation of 0.12 mol of ^{32}P/mol of tubulin whereas phosphorylation of undigested tubulin permits incorporation of about 0.08 mol of ^{32}P/mol of tubulin. The difference between these two values, 0.04 mol of ^{32}P/mol of tubulin, represents the amount of phosphate that was removed by the phosphatase digestion and is much smaller than the total tubulin phosphorylation which we are reporting

here; it is, in fact, smaller than our standard deviations. Even if we assume that the phosphate added in the rephosphorylation experiment was only added to β_{III}, this would give a stoichiometry of labeling of the $\alpha\beta_{III}$ dimer of 0.16 mol/mol, which is 11% of what we find is the total phosphate content of the $\alpha\beta_{III}$ dimer. It seems, therefore, that the phosphate on the β_{III} subunit is not very susceptible to digestion by a variety of phosphatases under the conditions used by Serrano et al. (1987).

The function of β_{III} phosphorylation is not yet known. Our results suggest that the dephosphorylation does not affect the assembly of pure tubulin induced by glycerol and $MgCl_2$. Removal of the phosphate inhibits the ability to polymerize to microtubules in the presence of MAP 2. The fact that calyculin A pretreatment abolishes the inhibition of assembly indicates that the inhibition is due to the phosphatase activity and not to a hypothetical contamination with another enzyme, perhaps a protease. Also, the fact that examination of silver-stained gels of phosphatase-treated and untreated tubulin showed no proteolytic fragments argues against proteolysis. Thus, it is likely that the presence of the phosphate influences assembly, at least with MAP 2. It should be noted that removal of phosphate and inhibition of assembly were both partial effects. Conceivably, had all the phosphates been removed, perhaps assembly would have been inhibited completely. It is also possible, however, that only one residue was dephosphorylated by the phosphatase, perhaps completely, and that other phosphorylated residues were untouched. In such a case, one would argue that phosphorylation of this residue has a relatively small effect on the assembly and that the role of phosphorylation of the other residue is still unclear.

Our results suggest that the phosphate residue may be involved with the tubulin−MAP 2 interaction. However, other interpretations are possible. A close look at Figures 2 and 3 will reveal that a substantially higher percentage of $\alpha\beta_{III}$ polymerized in the presence of glycerol and $MgCl_2$ than in the presence of MAP 2. This is consistent with the results of an experiment (not shown) in which $\alpha\beta_{III}$ was polymerized to steady state in the presence of MAP 2 and then, to one aliquot, glycerol and $MgCl_2$ were added to give final concentrations of 4 M and 6 mM, respectively; to the other aliquot was added an equal volume of glycerol-free buffer containing 0.5 mM $MgCl_2$. When the two samples were centrifuged and analyzed, the concentration of microtubules was 1.1 mg/mL in the glycerol-containing aliquot and 0.8 mg/mL in the control. This result suggests that glycerol can induce to polymerize a substantial fraction of the tubulin sample that is not able to assemble with MAP 2 alone. We also did the converse experiment in which $\alpha\beta_{III}$ was assembled to steady state in the glycerol−Mg buffer and then to one aliquot was added MAP 2 and to the other an equal volume of buffer only. The concentrations of microtubules in the two samples were, respectively, 1.1 mg/mL and 1.0 mg/mL, suggesting that addition of MAP 2 polymerized a much smaller additional fraction of the population than did the glycerol−Mg alone. Thus, the possibility exists that dephosphorylation of tubulin may cause it to decay more rapidly and lose its ability to assemble, as opposed to the model whereby the phosphorylated residue plays a direct role in assembly. It is conceivable that the glycerol−Mg buffer could then "rescue" some of this tubulin from decay and allow it to polymerize, hence masking the effect of dephosphorylation.

If we assume that the phosphate group has a specific assembly-related function, then one can imagine three possible ways in which a phosphate group on Ser^{444} or Tyr^{437} could play a role in microtubule assembly. First, we should remember that the C-terminal regions of the unmodified β_I, β_{II}, β_{IV}, and β_V isotypes are highly negatively charged. To be exact, from residue 437 to the C-terminus, β_I has 5 negatively charged residues and 4 uncharged; β_{II} has 7 negative residues and 3 uncharged; β_{IV} has 7 negative residues and 4 uncharged; β_V has 8 negative residues and 3 uncharged. In striking contrast, β_{III} has 7 negative residues, 1 positive, and 6 uncharged (Alexander et al., 1991). Another way to describe this is to state the total negative charge in this region as a percentage of the total number of residues from 437 on. For β_I, β_{II}, β_{IV}, and β_V , these values are, respectively, 56%, 70%, 64%, and 73%, whereas for β_{III} the value is 43%.[3] It is likely that the C-terminal region of β-tubulin interacts with MAPs to regulate assembly (Paschal et al., 1989; Cross et al., 1991). These interactions appear to include electrostatic bonds between negatively charged residues in tubulin and lysines or arginines in MAP 2 (Kotani et al., 1990). Conceivably, the unmodified β_{III}, having a less negative C-terminus, would be less able to interact with MAPs. Addition of negatively charged phosphate could perhaps compensate for the less negative amino acid sequence in this region of β_{III} and permit a tighter interaction with the MAPs and, hence, increased assembly.

The second possibility correlates the phosphorylation of Ser^{444} or Tyr^{437} to posttranslational glutamylation of Glu^{435}. It is known that all the monoglutamylated species of β_{III} are phosphorylated (Alexander et al., 1991) and that glutamylation of α- and β-tubulins increases their binding to tau or MAP 2 (Boucher et al., 1994). It has been demonstrated, though indirectly, that progressive glutamylation of α- and β-tubulins exerts a chain-length-dependent structural change in tubulin which modulates the affinity of C-terminal regions of α- and β- tubulins for tau or MAP 2 (Boucher et al., 1994). In other words, if the posttranslational incorporation of an additional 1−6 negative charges to the already acidic C-termini of α- and β-tubulins can modulate the tubulin−MAP 2 interaction, it is conceivable that electrostatic repulsion between the additional glutamates and a nearby phosphate could further change the orientation of the polyglutamate side chain and somehow alter the binding of MAP 2 to tubulin and thus regulate microtubule assembly. Removal of the phosphate would reverse this alteration.

A third possibility to consider is based on the observation of Burns and Surridge (1990) that there is a reciprocal relationship between position 437 and positions 217 and 218

[3] The β_I, β_{II}, β_{III}, and β_{IV} isotypes are all known to occur in bovine brain (Banerjee et al., 1988); hence, it is valid to compare their structural properties when speculating about their functional significance. Although the β_V isotype does not appear to occur in avian brains (Lopata & Cleveland, 1987), its distribution in mammalian tissues is still uncertain (Ahmad et al., 1991). Since it is conceivable that β_V also occurs in mammalian brains, we have included it in our comparison. Interestingly, the highly divergent mammalian β_{VI} isotype, which appears to be restricted to hematopoietic tissues such as platelets (Wang et al., 1986), resembles β_{III} in that its C-terminal region from position 437 on contains 9 negative, 2 positive, and 4 uncharged residues, giving a negative charge percentage of 47%. The avian β_{VI}, which occurs in erythrocytes, has, in the corresponding region, 5 negative, 1 positive, and 6 uncharged residues, with a negative charge percentage of 33% (Sullivan, 1988). It is interesting that turkey erythrocyte β_{VI} also resembles β_{III} in being phosphorylated, at Ser^{441} (Rüdiger & Weber, 1993).

in the β isotypes. If position 437 is phenylalanine, then positions 217 and 218 are both threonine; if position 437 is tyrosine, then 217 and 218 are not threonine. Burns and Surridge (1990) propose that there is some kind of interaction between position 437 and positions 217−218. If that is the case, the presence of a phosphate at position 437 would certainly influence this interaction. Perhaps even a phosphate at position 444 could influence this as well. Since the C-terminal region is known to play a major role in regulating assembly, acting as an endogenous inhibitor (Serrano et al., 1984), phosphorylation of a residue that may be involved in interactions of the C-terminal region with other parts of the protein may have a role in regulating assembly.

Our results show that dephosphorylation of β_{III} influences microtubule assembly. Although we cannot be certain about the mechanism, our results raise the possibility that phosphorylation of β_{III} may have a regulatory role in microtubule assembly.

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