Biochemical Dissection of the Role of the One-Kilodalton Carboxyl-Terminal Moiety of Tubulin in Its Assembly into Microtubules†

Juan C. Vera, Coralia I. Rivas, and Ricardo B. Maccioni*

Department of Biochemistry, Biophysics and Genetics, University of Colorado Health Sciences Center, Denver, Colorado 80262, and International Center for Cancer and Developmental Biology, Santiago, Chile

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ABSTRACT: The 4-kDa C-terminal domain of both tubulin subunits plays a major role in the regulation of microtubule assembly [Serrano et al. (1984) *Biochemistry 23*, 4675]. Controlled proteolysis of tubulin with subtilisin produces the selective cleavage of this 4-kDa moiety from α- and β-tubulin with a concomitant enhancement of the assembly. Here we show that gradual removal of the last six to eight amino acid residues of the C-terminal region of α and β subunits by an exopeptidase, carboxypeptidase Y, produces a modified protein (C-tubulin) without relieving the modulatory effect of the C-terminal domain and the usual need of MAPs for microtubule assembly. Actually, treatment with this proteolytic enzyme did not change tubulin assembly as promoted by either MAP-2, taxol, $MgCl_2$, dimethyl sulfoxide, or glycerol. The critical concentration for the assembly of C-tubulin remained the same as that for the unmodified tubulin control. Microtubule-associated proteins MAP-2 and τ incorporated into C-tubulin polymers. Clearly, pure C-tubulin did not assemble in the absence of MAPs or without addition of assembly-promoting compounds. However, proteolysis with the exopeptidase induced changes in tubulin conformation as assessed by biophysical methods and double-limited proteolysis. The cleavage with subtilisin after carboxypeptidase digestion did not result in enhancement of the assembly to the levels observed after the treatment of native tubulin with subtilisin. Interestingly, Ca^{2+} ions affected neither C-tubulin assembly nor depolymerized microtubules assembled from C-tubulin. These findings indicate that the very end C-terminal moiety, which has been shown to be the region that defines tubulin isotypes, is not directly involved in the selective interactions of MAPs. Instead, this region appears to be essential for the conformation of native tubulin and also for its sensitivity to Ca^{2+} inhibition.

Recent studies from our laboratory have demonstrated that the carboxyl-terminal moiety of tubulin subunits plays a major role in modulating its assembly into microtubules (Serrano et al., 1984a; Maccioni et al., 1985a). Further support to this findings has been provided by interesting studies on the biochemical (Bhattacharyya et al., 1985) and molecular biological (Lewis et al., 1985; Sullivan & Cleveland, 1986; Lopata & Cleveland, 1987) aspects of this regulatory domain. Controlled proteolysis of tubulin with subtilisin results in the cleavage of both α and β subunits yielding the cleaved tubulin heterodimer (S-tubulin)[1] and two 4-kDa fragments containing the carboxyl-terminal regulatory domain of tubulin subunits (Serrano et al., 1984b). S-Tubulin has an increased propensity to self-assemble as compared with tubulin, forming microtubule bundles and hooked polymers. Such assembly without the usual need of MAPs indicates that the 4-kDa tubulin domain hinders the interactions responsible for tubulin self-association. Interaction of MAPs with the acidic 4-kDa domain of tubulin appears to relieve the hindering effect, thus stimulating the assembly (Serrano et al., 1984a,b, 1985).

These studies along with previous findings on the structure of S-tubulin and the 4-kDa domain (Maccioni et al., 1985a,b) led us to postulate the existence of two major functionally significant regions in the tubulin molecule: the carboxyl-terminal regulatory domain and a larger region containing the sites for the interaction of tubulin subunits as well as for the modulatory ligands involved in the assembly. In order to gain information on the substructure of this regulatory domain, which is important to understand the interaction of tubulin with MAPs and the modulation of tubulin assembly, we have analyzed the effects of limited proteolysis of tubulin with carboxypeptidase Y on the conformation and assembly of tubulin and its interaction with microtubule-associated proteins.

Materials and Methods

Preparation of Microtubules, Tubulin, and MAPs. Microtubule protein was prepared from cow brains by two temperature-dependent cycles of assembly and disassembly (Shelanski et al., 1973) and stored as pellets at −70 °C. Immediately before use, the microtubular pellets were resuspended in 0.1 M Mes (pH 6.8), 1 mM EGTA, and 1.5 mM $MgCl_2$ (assembly buffer), and a third cycle of assembly and disassembly was performed (Maccioni et al., 1985a) following which tubulin depleted of MAPs was obtained by phosphocellulose chromatography (Weingarten et al., 1975). Tubulin preparations used in this study were >96% pure. Total MAPs were obtined after elution of the protein retained in the phosphocellulose column with 0.7 M NaCl. The microtubule-associated proteins MAP-2 and τ were isolated after boiling the resuspended microtubular protein followed by ammonium sulfate fractionation and chromatography in Se-

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* Address correspondence to this author at the Department of Biochemistry, Biophysics and Genetics, University of Colorado Health Sciences Center B-121, 4200 E. 9th Ave., Denver, CO 80262.

[1] Abbreviations: C-tubulin, carboxypeptidase Y cleaved tubulin heterodimer; C-terminal, carboxyl terminal; CD, circular dichroism; DMSO, dimethyl sulfoxide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; GTP, guanosine 5′-triphosphate; Mes, 2-(*N*-morpholino)ethanesulfonic acid; MAPs, microtubule-associated proteins; SDS, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride; S-tubulin, subtilisin-cleaved tubulin dimer.

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pharose 6B gels as described in previous reports (Herzog & Weber, 1978). Purity was confirmed in all cases by SDS–acrylamide gel electrophoresis.

Protein Determinations. Protein concentrations of tubulin samples were determined spectrophotometrically using $A^{0.1\%}_{280nm} = 1.15$. MAP-2 concentrations were determined spectrophotometrically using an absorption coefficient $A^{0.1\%}_{275nm} = 0.33$ (Hernandez et al., 1986). In some experiments, the amount of protein was quantitated by turbidimetry. For these determinations, protein solutions containing 2–40 μg of protein in a final volume of 0.1 mL were pipetted into 3 × 100 mm test tubes followed by the immediate addition of 0.5 mL of 20% trichloroacetic acid. The turbidity at 340 nm was measured after 30-min incubation at room temperature. A linear relationship between turbidity at 340 nm and protein concentration is observed in the range 2–40 μg of protein, using bovine serum albumin or purified tubulin as standards (Vera, 1988).

Assembly Assays. (*A*) *The turbidimetric method* (Gaskin et al., 1974) was employed to monitor the kinetics of tubulin assembly in mixtures containing purified tubulin and MAP-2 or τ and to assess the assembly activities of purified tubulin preparations and tubulin cleaved with the different proteases. The assays with appropriate blanks were carried out in a Beckman Model 25 spectrophotometer equipped with a chart recorder. Tubulin (2–3 mg/mL) in assembly buffer containing either 10% dimethyl sulfoxide, MAP-2, or τ (0.4–0.55 mg/mL) was allowed to assemble after the addition of 1 mM GTP to the assay mixture. The time course of the turbidity increase at 37 °C was recorded at 340 nm starting immediately after the addition of GTP; the recording was stopped 3–10 min after the plateau was reached. Cold reversibility was tested by cooling the assembly mixture to 0 °C for 10 min and remeasuring the turbidity.

(*B*) *The sedimentation method* (Serrano et al., 1984a) was employed for quantification of the extent of assembly in all but few of the studies using cleaved tubulin and the experiments on the time course of the assembly following limited proteolysis. Assembly was initiated by addition of 1 mM GTP to tubulin in assembly buffer containing either 10% dimethyl sulfoxide, 15 μM taxol, 4 M glycerol, 10 mM Mg^{2+}, MAP-2, or τ and allowed to proceed for 30 min at 37 °C. Blanks were treated identically except for the omission of GTP. The assembled protein was sedimented at room temperature in a Beckman Airfuge for 15 min at 130000*g*. The protein content of the pellets was quantitated by the procedure of Lowry et al. (1951) or by turbidimetry at 340 nm using trichloroacetic acid (see above). The extent of assembly was obtained by subtracting the very small amounts of sedimented protein in the blanks.

Tubulin Cleavage with Carboxypeptidase Y. Controlled cleavage with carboxypeptidase Y was performed by digestion at 32 °C for different periods of time. Proteolytic digestion was terminated immediately after recovering samples from the digestion mixture by addition of a mixture of phenylmethanesulfonyl fluoride (PMSF) to a final concentration of 0.2 mM and aprotinin and soybean trypsin inhibitor to final concentrations of 20 μg/mL.

Electrophoresis. Digested tubulin samples were adjusted to 1% sodium dodecyl sulfate (SDS), 3% 2-mercaptoethanol, 2 M glycerol, and 0.01% bromphenol blue and boiled for 2 min. Samples were analyzed by SDS–polyacrylamide gel electrophoresis in gels with 5–15% acrylamide gradients. A constant current of 20 mA/gel was used in all electrophoresis experiments. After electrophoresis, gels were stained with 0.2% Coomassie brilliant blue R in methanol/acetic acid/water (50:10:40 v/v).

To avoid comigration of τ and tubulin during the electrophoretic fractionation, samples containing τ were boiled for 5 min (Herzog & Weber, 1978) prior to electrophoresis in order to selectively precipitate tubulin.

Electron Microscopy. The morphology of the assembly products from proteolytically cleaved tubulin and undigested tubulin was examined after negative staining of polymer suspensions with 1% uranyl acetate. Thin sections from the polymer pellets were also prepared as indicated by Serrano et al. (1984b) and observed under a Philips 300 electron microscope.

pH-Dependent Binding Analysis. The pH-dependent binding analysis was performed essentially as described (Yang & Langer, 1985) utilizing a strong ion exchanger, SP-Sephadex C-50. We modified the original procedure by including 6 M urea in all buffers and scaled-down the method to use lower amounts of protein.

Amino Acid Analysis. The analysis was performed by determination of amino acids released from purified tubulin after digestion with the exopeptidase. Tubulin (2–5 mg/mL) in 0.05 M Mes was subjected to proteolysis at 32 °C with freshly dissolved carboxypeptidase Y (1% w/w with respect to tubulin). Aliquots were withdrawn from the digested tubulin solution at 0, 15, 30, 60, and 120 min, and digestion was terminated by addition of sulfosalicylic acid at a final concentration of 5% w/v. The released amino acids were quantitated by amino acid analysis in triplicate samples, using a two-column amino acid analyzer, Beckman 120-C.

Physical Methods. CD spectra were recorded on a Cary Model 60 spectropolarimeter with a Model 6001 CD attachment, the sample compartment being maintained at 32 °C. Slits were programmed to yield a 15-Å bandwidth at each wavelength. The concentrations of tubulin and carboxypeptidase Y treated tubulin were 0.3–0.4 mg/mL. Path lengths were 5–0.5 mm depending upon wavelength range. Mean residue ellipticities, $[\theta]_{mrw}$ (degrees centimeter squared per decimole), were calculated in the usual fashion. A value of 115 was used for the mean residue weight.

Fluorescence spectra were recorded on a Farrand Mark I spectrofluorometer fitted with a constant-temperature cell holder connected to a constant-temperature circulating water bath. The temperature was regulated at 27 °C. The excitation wavelength was 278 nm, and slits of 2.5 nm were used. Measurements were made at a protein concentration 0.1 mg/mL in 3-mL cuvettes.

Results

Effect of Carboxypeptidase Y Treatment on Tubulin Assembly. Tubulin was subjected to controlled proteolysis with an exopeptidase, carboxypeptidase Y. In time course digestion experiments using 1% carboxypeptidase Y, tubulin assembly was not affected at all (Figure 1A). Tubulin samples obtained at different time intervals from the digestion mixture were induced to assemble in the presence of either 15 μM taxol, 4 M glycerol, or 10 mM $MgCl_2$ or after addition of MAP-2. No effect of proteolysis on the assembly under all these experimental conditions was observed. Similar results were obtained when carboxypeptidase Y treated tubulin was induced to assemble in the presence of 10% DMSO or τ (data not shown). Digestion of purified tubulin with 2% carboxypeptidase did not have an effect either. An analysis of the critical concentration ([Cr]) for tubulin assembly as induced by these assembly-promoting compounds indicated that this value ([Cr]) remained the same after proteolysis as shown in the plots of

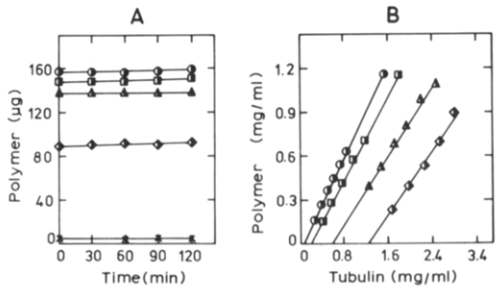

FIGURE 1: (A) Time course of the assembly of tubulin and carboxypeptidase Y digested tubulin. Tubulin (97% purity) was treated with carboxypeptidase Y (1% w/w with respect to tubulin) at 32 °C, and aliquots were obtained at the times indicated and induced to assemble with either 15 μM taxol (●), 4 M glycerol (▲), 10 mM $MgCl_2$ (♦), or 0.8 mg/mL MAP-2 (■) or in the absence of assembly-promoting compounds (×). Controls correspond to tubulin incubated at 32 °C in the absence of the exopeptidase and induced to assemble with taxol (○), glycerol (△), 10 mM $MgCl_2$ (◇), or MAP-2 (□). The tubulin concentration was 1.6 mg/mL in the experiments with taxol and MAP-2, 2.4 mg/mL in the presence of glycerol and $MgCl_2$, and 2.4 mg/mL in the absence of assembly-promoting compounds. The volume of the assembly assay was 120 μL, and the degree of assembly was determined by the sedimentation procedure. (B) Plots of the amount of microtubules formed vs the total tubulin concentration, for samples of tubulin treated with carboxypeptidase Y (1% w/w with respect to tubulin) at 32 °C for 120 min. C-Tubulin was induced to assemble with either 15 μM taxol (●), 4 M glycerol (▲), 10 mM $MgCl_2$ (♦), or MAP-2 (1:6 molar ratio with respect to tubulin) (■). Controls correspond to tubulin incubated at 32 °C for 120 min in the absence of the exopeptidase and induced to assemble with taxol (○), glycerol (△), $MgCl_2$ (◇), or MAP-2 (□). After centrifugation, microtubular pellets were resuspended in 120 μL of 0.1 M Mes (pH 6.8), and the protein was determined as indicated under Materials and Methods.

amount polymer vs tubulin concentration (Figure 1B). In addition, carboxypeptidase Y treated tubulin was not able to self-assemble in the absence of assembly-promoting compounds (Figure 1A), indicating that proteolysis with the exopeptidase does not relieve the hindering effect of the C-terminal domain as does subtilisin proteolysis of tubulin (Maccioni et al., 1985a,b).

MAPs Incorporation into Polymers. Incorporation of MAPs and τ into polymers obtained from digested and undigested tubulin was analyzed by gel electrophoresis. Polymers from tubulin digested with carboxypeptidase for various times and induced to assemble in the presence of MAP-2 contained the associated protein as well as in undigested controls (Figure 2). As indicated in the lower part of Figure 2, the relative proportions of tubulin and MAP-2 in the polymer did not change after treatment with carboxypeptidase Y. Thus, proteolysis with the exopeptidase did not change the ability of assembling tubulin to incorporate MAP-2 into the polymers. τ also incorporated into the polymers obtained from digestion products of tubulin treated with carboxypeptidase Y at these time intervals, and the relative proportions of tubulin and τ in the polymer did not change after treatment with carboxypeptidase Y (data not shown).

Structure of Polymers Obtained from Cleaved Tubulin. As seen in Figure 3, carboxypeptidase Y treated tubulin assemblies into polymers with characteristics of microtubules. Some microtubules with frayed ends were observed after assembly of tubulin exposed to the carboxypeptidase Y at the highest digestion times. Samples in which assembled microtubules were subjected to cold were also examined in the electron microscope and showed depolymerization as in microtubules formed by undigested tubulin. The morphological appearance and the amount of polymers observed after τ, taxol-, or DMSO-induced assembly of C-tubulin were comparable with those obtained from unmodified tubulin.

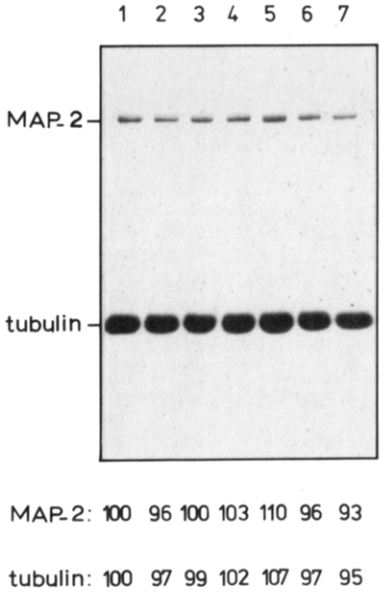

FIGURE 2: Incorporation of MAP-2 into polymers obtained from tubulin digested with carboxypeptidase Y (1% w/w with respect to tubulin) for 0 min (lane 1), 30 min (lane 2), 60 min (lane 3), 90 min (lane 4), and 120 min (lane 5) as analyzed by SDS–polyacrylamide gel electrophoresis. Controls correspond to polymers obtained from tubulin incubated for 60 min (lane 6) and 120 min (lane 7) in the absence of the exopeptidase. The concentrations of tubulin and MAP-2 in the assembly assay were 1.6 and 0.8 mg/mL, respectively. The assembled polymers were sedimented by centrifugation at 130000*g*; the pellets were resuspended in 0.05 M Tris-HCl (pH 6.8) containing 0.5% SDS and 1% 2-mercaptoethanol. Equivalent aliquots of each sample were analyzed by electrophoresis. After being stained with Coomassie blue, the protein bands corresponding to MAP-2 and tubulin from the different lanes were cut, and the bound dye was eluted and quantitated by the absorbance at 590 nm (Ball, 1986). The results obtained are shown in the lower half of the figure and are normalized by using the respective controls as reference (lane 1, zero time and no digestion, 100%).

Table I: Amino Acid Composition of Tubulin Residues Hydrolyzed by Carboxypeptidase

	residues/mol of heterodimer		
amino acid	amino acid analysis	derived from sequence[a]	derived from sequence[b]
Lys	0.21	0.25	0.25
Asp	0.75	1.25	1.25
Ser	0.18	0.50	0.50
Glu + Gln	9.20	8.25	9.75
Gly	2.80	2.00	2.25
Ala	1.00	1.25	1.25
Val[c]		0.25	0.25

[a] From sequence of brain C-terminal seven residues of α-tubulin and β-tubulin isotypes, assuming equal distribution of isotypes (Gu et al., 1988; Pratt & Cleveland, 1988; Ludueña et al., 1988). The expected amino acid compositions do not consider removal of tyrosine from the small fraction of the tyrosinated $M\alpha_{1/2}$-tubulin isotype (the $M\alpha_4$-tubulin isotype does not contain tyrosine). [b] From sequence of C-terminal eight residues from α-tubulin and each β-tubulin isotype, assuming equal distribution. [c] Traces.

Amino Acid Residues Removed by Carboxypeptidase Y. The C-terminal amino acid sequences of mammalian brain α- and β-tubulins are shown in Figure 4. The amino acid analysis of supernatants obtained after sulfosalicylic acid precipitation of digested tubulin samples (Table I) indicated that carboxypeptidase Y digestion for 120 min resulted in removal of six to eight amino acid residues from the carboxyl-terminal moiety of both α and β subunits. The results of the amino acid analysis are in good agreement with the ex-

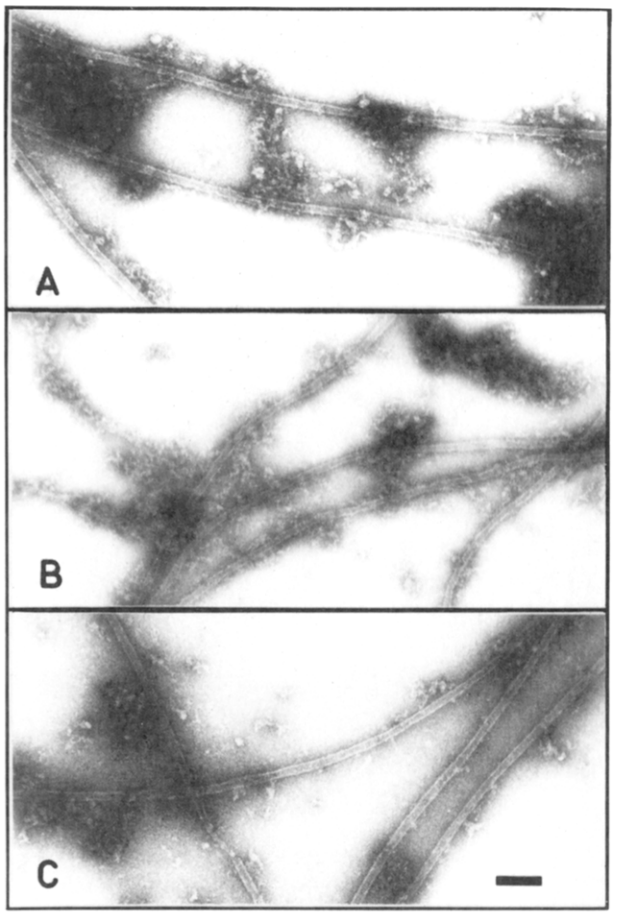

FIGURE 3: Electron microscopy of polymers obtained by assembly of tubulin cleaved with carboxypeptidase Y. Tubulin (1.2 mg/mL) was assembled in the presence of MAP-2 (0.4 mg/mL). Polymer obtained from tubulin digested with carboxypeptidase Y for 60 min (B) and 120 min (C). Microtubules from undigested tubulin control (A). Bar corresponds to 0.1 μm.

α-tubulin isotypes:

	420	430	440
Mα1/2	EAREDMAALE	KDYEEVGVDSVE	GEGEEEGEEY
Mα4	EAREDMAALEKDYEEVGVDI		DSYEDEDEGEE

β-tubulin isotypes:

	420	430	440
Type I	SEYQQYQDATAEEEEDFGEEAEEEA		
Type II	SEYQQYQDATADEQGEFEEEGEEDEA		
Type III	SEYQQYQDATAEEEGEMYEDDEEESESQGPK		
Type IV	SEYQQYQDATAEEEGEFEEEAEEEVA		

FIGURE 4: Carboxyl-terminal sequences of mammalian brain α- and β-tubulin isotypes. Sequence data for α-tubulin isotypes were obtained from Gu et al. (1988), and those for β isotypes were obtained from Pratt and Cleveland (1988) and Ludueña et al. (1988). The underlined sequences correspond to the highly conserved regions in α- and β-tubulin isotypes involved in the binding of MAPs (Maccioni et al., 1987, 1988; Vera et al., 1988; Rivas et al., 1988).

pected amino acid content derived from the sequence of the last six to eight amino acids from α-tubulin and from the respective β-tubulin isotypes. Differences were found in the content of aspartic acid (0.75 residue/mol of tubulin dimer compared with an expected number of 1.25 residues/mol), in glycine (2.8 residues/mol vs an expected analysis of 2–2.25 residues/mol), and in valine (traces versus 0.25 residue/mol). Tubulin labeled with [^{3}H]tyrosine in its α subunit (Serrano et al., 1984a) was also treated with carboxypeptidase Y for 120 min, and a 100% release of the radiolabeled C-terminal amino acid was observed. Furthermore, the polymer obtained from carboxypeptidase Y treated ^{3}H-tyrosinated tubulin did not incorporate radioactivity.

The gradual removal of the very end C-terminal residues by carboxypeptidase Y was confirmed by an experiment in which native tubulin and its digestion product were compared with respect to their capacity to bind to sulfopropyl (SP) Sephadex in a pH-dependent fashion (Yang & Langer, 1985). A plot of unretained protein as a function of pH (in the range of pH 4–8) shows that the digestion of tubulin with carboxypeptidase Y was correlated with a shift in the sigmoidal curve of the unretained protein vs pH toward higher pH values as compared with unmodified tubulin. The middle value of the titration curve was shifted from pH 5.5 to pH 6.0. Polymers obtained from C-tubulin and unmodified tubulin control were depolymerized, and the pH profile of SP-Sephadex-adsorbed protein was analyzed accordingly. The pH binding curve of the polymer obtained with carboxypeptidase Y treated tubulin exhibited a similar shift toward basic pH values as compared with unmodified controls. Electrophoresis experiments under nondenaturing conditions (Sackett et al., 1985) showed a differential migration of digested tubulin as compared with the untreated controls (data not shown). No traces of undigested tubulin were detected in the carboxypeptidase-treated samples, which does not exclude the possibility that a minor fraction of tubulin may not be digested completely.

Conformational Studies of Cleaved Tubulin. The conformation of the cleaved product from carboxypeptidase Y treatment of tubulin was examined by fluorescence spectroscopy and circular dichroism. The emission spectrum showed that fluorescence quenching accompanied tubulin proteolysis with 1% carboxypeptidase Y. An increase in the maximum at 340 nm of the emission spectrum after 30-min proteolysis was followed by a gradual decrease in the fluorescence maximum as carboxypeptidase Y digestion progressed over a period of 120 min. In addition, CD spectroscopy showed that carboxypeptidase Y cleavage of tubulin for 120 min resulted in a conformational change as revealed by a decrease in its helical content. The spectrum of carboxypeptidase Y digested tubulin showed two negative extremes, the stronger one at 219 nm ($[\theta]_{mrw}$ = 8050) and a slightly weaker minimum at 211 nm ($[\theta]_{mrw}$ = 7550). Untreated controls showed a stronger extreme at 221 nm ($[\theta]_{mrw}$ = 11 700) and a slightly weaker minimum at 210 nm ($[\theta]_{mrw}$ = 11 050).

Limited proteolysis has proven to be useful to analyze tubulin conformation (Maccioni & Seeds, 1983). Tubulin was subjected to double-limited proteolysis with carboxypeptidase Y followed by subtilisin. The assembly analysis showed that digestion with subtilisin after carboxypeptidase Y proteolysis did not result in enhancement of the assembly to the levels observed after proteolysis of native tubulin with subtilisin (Table II). The sedimentation method was used to quantitate the assembly, since the turbidimetric procedure is not appropriate due to the aberrant nature of the subtilisin-treated tubulin polymers. Electrophoresis experiments showed that carboxypeptidase Y pretreatment of tubulin stimulates subtilisin cleavage at a second site with formation of fragments of 32 and 20 kDa in addition to the cleavage releasing the 4-kDa peptide. This experiment supports the observation that carboxypeptidase Y proteolysis induces a conformational alteration on tubulin.

Sensitivity to Ca^{2+} Ion Inhibition. Assembly of C-tubulin was not affected by the presence of Ca^{2+} ions (100 μM) (Figure 5B) under conditions that inhibited significantly the

Table II: Assembly of Tubulin Subjected to Double Digestion with Carboxypeptidase Y and Subtilisin

tubulin derivative[a]	net assembly (μg of polymer)
S-tubulin	174.0 ± 5.5
subtilisin-cleaved[b] C-tubulin	29.5 ± 4.1
C-tubulin	8.6 ± 0.7
tubulin	12.5 ± 1.8

[a] Tubulin (>96% purity) and proteolytic derivatives of tubulin were at a final concentration of 2.1 mg/mL. Assembly was assayed by the sedimentation procedure in 0.18-mL aliquots of each protein. Other conditions for assembly are described under Materials and Methods. [b] Tubulin was subjected to double-limited proteolysis: After carboxypeptidase Y cleavage (1% w/w) at 32 °C for 120 min, the proteolytic product (C-tubulin) was passed through Sephadex G-25 equilibrated in 0.1 M Mes, pH 6.8, concentrated, adjusted to 1 mM Mg^{2+}, and digested with 1.2% w/w subtilisin for 30 min at 30 °C. Digestion was terminated by addition of 0.1 mM PMSF, soybean trypsin inhibitor, and aprotinin to final concentrations of 20 μg/mL.

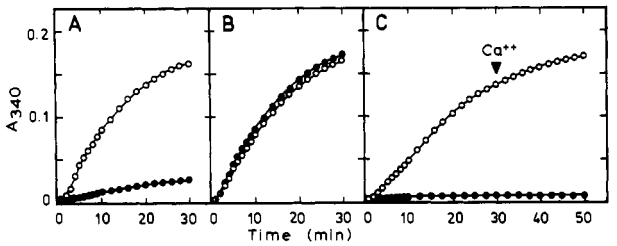

FIGURE 5: Effect of Ca^{2+} ions on tubulin and C-tubulin assembly and on preassembled polymers from C-tubulin. (A) Tubulin (1.4 mg/mL) was induced to assemble with 8% DMSO in the absence (○) or in the presence (●) of 0.1 mM Ca^{2+}. (B) Tubulin (1.4 mg/mL) digested for 120 min with 1% carboxypeptidase Y was induced to assemble with DMSO in the absence (○) or in the presence (●) of 0.1 mM Ca^{2+} ions. (C) Carboxypeptidase Y digested tubulin (1.4 mg/mL) was induced to assemble with 0.48 mg/mL τ factor (○). Ca^{2+} (0.1 mM) was added after 30 min (arrowhead) and the recording of the turbidity increase continued for an additional time interval. Control of tubulin incubated under assembly conditions but in the absence of τ (●).

in vitro assembly or unmodified tubulin controls (Figure 5A). In addition, microtubules preassembled from C-tubulin were insensitive to inhibition by Ca^{2+} ions (Figure 5C) at a concentration (100 μM) that induces depolymerization of microtubules from unmodified tubulin.

Discussion

Removal of the very acidic extreme C-terminal residues of both tubulin subunits by treatment with carboxypeptidase Y does not affect its ability to self-associate into microtubules in the presence of assembly-promoting compounds, even though a conformational change was evidenced as a result of the proteolysis. The determination of the residues cleaved by carboxypeptidase Y (1% w/w, 32 °C, 120 min) showed the release of glutamic acid, glycine, aspartic acid, alanine, lysine, serine, and traces of valine in stoichiometric relationships that are in good agreement with the expected amino acid content from the sequence of at least six (or up to eight) residues of the C-terminal end, considering cleavage of both α- and β-tubulin. If 7 amino acid residues were to be released from both the α and β subunits, we should expect to find 8.25 glutamic acid + glutamine residues, 2.00 glycines, 1.25 alanines, 0.50 serine, and 0.25 lysine residues, which is in approximation with the amino acid analysis data. A salient difference found between the amino acid analysis and the expected amino acid content from the sequences of the β-tubulin isotypes is the very low level, difficult to quantitate, of valine. One possible interpretation is a diminished digestion of type IV β-tubulin isotypes. This may also result from possible differences in the sequences and/or level of expression of cow brain β-tubulin isotypes, as compared with the known mouse and human sequences. The latter explanation may be also valid for the differences in aspartic acid content (0.75 residue/mol of dimer versus an expected number of 1.25 residues/mol). Since a differential digestion of α-tubulin isotypes may occur, the possibility exists that the digestion of $M\alpha_4$-tubulin might proceed with six steps into the sequence, thus accounting for the lower content of aspartic acid. As indicated, our results do not imply that all α-tubulin and the β-tubulin isotypes were digested the same number of steps. It is important to point out that the amino acid contents derived from the sequences were obtained by assuming equal distribution of brain isotypes. The exact levels of α- and β-tubulin isotypes in cow brain are not known. All these factors may explain minor differences in the amino acid composition with respect to that derived from the available sequence data (Pratt & Cleveland, 1988; Ludueña et al., 1988; Gu et al., 1988). Further support for the removal of an approximately 1-kDa fragment from tubulin subunits was provided by the differential binding to SP-Sephadex of modified and unmodified tubulin. The seemingly proportional shifting of carboxypeptidase Y treated tubulin toward the basic pH zone within the entire range of pHs examined also suggests that a similar loss of acidic residues from both tubulin subunits resulted from proteolysis with the exopeptidase.

Selective cleavage of tubulin subunits with subtilisin results in removal of the 4-kDa C-terminal domain, thus relieving the hindering effect of this domain (Serrano et al., 1984b; Maccioni et al., 1985a). In this context, it is interesting that removal of the very acidic extreme segment of the C-terminal region by carboxypeptidase Y digestion does not contribute at all to the relief of the hindering effect. This observation along with studies using proteases that cleaved tubulin distally and proximately to the subtilisin site (α E^{417}–F^{418}; β E^{407}–F^{408}) indicates that cleavage at or near the subtilisin site is critical in order to overcome the normal barrier to self-assembly. The lack of a modulatory effect of the ~1-kDa C-terminal moiety on MAP-mediated tubulin assembly is also supported by the observation that purified MAP-2 and τ incorporate into microtubules formed from tubulin lacking the extreme ~1-kDa subdomain. This finding, taken along with our studies using synthetic tubulin peptides (Maccioni et al., 1987, 1988) and studies using MAP-reacting antiidiotypic antibodies (Rivas et al., 1988), strongly indicates that the extreme C-terminal moiety of tubulin (~1 kDa) is not directly involved in the selective interaction of tubulin with MAP-2 and τ. These findings are also in agreement with the observation that a monoclonal antibody interacting with the tubulin domain around the C-terminal tyrosine (Kilmartin et al., 1982) does not inhibit MAP-induced microtubule assembly (Wehland et al., 1983) and our recent finding that antibodies produced against the α (430–441) and β (422–434) synthetic tubulin peptides strongly inhibit the assembly (Vera et al., 1988). Transfection experiments reported recently (Fridovich-Keil et al., 1987) provide further support to our observations.

Despite the lack of effect on MAP-induced tubulin assembly and on tubulin's self-assembly capacity, limited proteolysis with carboxypeptidase Y produced a loss of sensitivity in tubulin assembly to the inhibition by Ca^{2+} ions. Microtubules obtained from C-tubulin, even though similar in morphology to normal microtubules, were also resistant to Ca^{2+} depolymerization in contrast to polymers obtained from unmodified tubulin. These data suggest that the very end C-terminal moiety is required for the inhibitory action of Ca^{2+} on tubulin assembly. This could be related to the conformational change resulting from

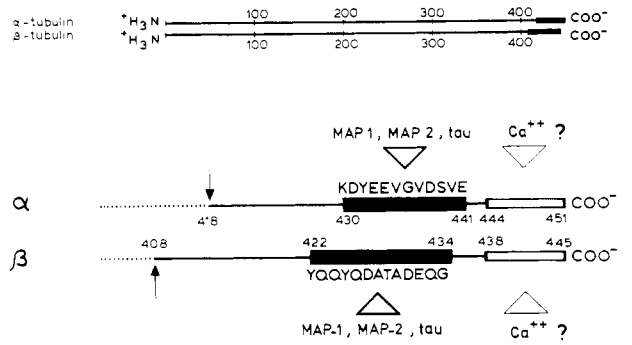

FIGURE 6: Schematic representation of the substructure of the 4-kDa C-terminal domain of tubulin subunits. (Top) Diagram of α- and β-tubulin subunits showing the C-terminal regulatory domain (filled boxes). (Bottom) Functionally significant subdomains within the 4-kDa regulatory moiety of α- and β-tubulin. The arrows indicate the sites for subtilisin cleavage (Maccioni et al., 1985a). The filled boxes represent the sequence of the low-homology region between α- and β-tubulin subunits directly involved in the selective binding of MAPs (Maccioni et al., 1987, 1988). The open boxes correspond to the last eight amino acid residues of both tubulin subunits removed by carboxypeptidase Y treatment. The identification of tubulin subdomains was based on the sequence data of porcine tubulin (Ponstingl et al., 1982).

the exopeptidase treatment or to a possible direct role of this very end C-terminal region in the interaction of Ca^{2+}. Studies by Serrano et al. (1986) have shown that the 4-kDa C-terminal domain contains the high-affinity Ca^{2+} binding site. In this context, it is interesting to point out that other Ca^{2+} binding proteins have high-homology regions as compared with the sequences of the last 10–15 C-terminal amino acids of α-tubulin (Serrano et al., 1986; Baudier & Cole, 1988).

The present findings taken together with previous results on the mapping of tubulin subdomains involved in MAP and Ca^{2+} interactions indicate that the 4-kDa regulatory domain can be visualized as a protein region composed of at least two major functionally significant subdomains: an internal region directly involved in the binding of MAPs and the extreme ~1-kDa C-terminal moiety which appears to be related to the conformational stability of tubulin and its sensitivity to Ca^{2+} inhibition (Figure 6). At the level of isotypes, however, the situation appears to be rather complex since it is possible that some specific tubulin isotypes may be responsible for Ca^{2+} inhibition (see below).

Soon after the initial finding (Serrano et al., 1983, 1984a,b) that a 4-kDa domain of the carboxyl-terminal region of tubulin appeared to be involved in the interaction of MAPs, studies at the genetic and molecular biological levels have been directed to the extreme C-terminal moiety containing the last 10–15 amino acid residues of tubulin subunits, which define tubulin isotypes (Lewis et al., 1985; Sullivan & Cleveland, 1986; Cleveland, 1987; Pratt & Cleveland, 1988). In the context of the present results, the analysis of brain tubulin isotypes can also shed light into the structural–functional aspects of the C-terminal region of tubulin. As discussed previously, the amino acid sequence between residues 422 and 434 of the β-tubulin subunit together with the sequence between amino acid residues 430 and 441 of α-tubulin defines or at least is part of the MAP binding domain of tubulin (Maccioni et al., 1987, 1988; Rivas et al., 1988; Vera et al., 1988). It is noteworthy that these sequences are highly conserved in all tubulin isotypes. Sequence differences among isotypes are localized in the last 10–15 amino acid residues (Lewis et al., 1985; Sullivan & Cleveland, 1986). Our results appear to indicate that digestion with carboxypeptidase Y occurs in all isotypes, with a range of six to eight hydrolysis steps into the sequence. In regard to MAPs interaction, this analysis along with the results that MAP binding remained unaffected after proteolysis with carboxypeptidase Y strongly suggests that the different isotypes may bind MAP-2 and τ. However, since we have shown a differential affinity of MAP-2 and τ for α- and β-tubulin subunits (Maccioni et al., 1987, 1988), it is also possible that tubulin isotypes may respond with differential affinities to these MAP components, a question that remains to be elucidated.

In regard to the effects of digestion with carboxypeptidase Y on Ca^{2+} inhibition, it is possible that this cation may bind preferentially to some specific isotypes and may even not bind to some of them. Since the five isotypes appear to be digested, it is difficult to ascertain the roles of each isotype component on calcium regulation of assembly. Despite the apparent lack of participation of the 1-kDa C-terminal moiety in the interaction of MAPs, this region appears to contribute to the native tubulin conformation and the inhibitory action of Ca^{2+}, of regulatory importance for microtubule assembly (Maccioni, 1985b; Serrano et al., 1986). This suggests that there could be some subtle functional differences among tubulin isotypes. In this context, it is worth mentioning that the specific phosphorylation of a β-tubulin isotype (type III) in differentiated neuroblastoma cells appears to be of regulatory significance (Ludueña et al., 1988).

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Self-Association of Calcium and Magnesium Complexes of Dentin Phosphophoryn†

Mary E. Marsh
Dental Science Institute, The University of Texas Health Science Center, Houston, Texas 77225
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ABSTRACT: Self-association of rat dentin phosphophoryn in the presence of calcium and magnesium ions was examined by chemical cross-linking and electron microscopy. Highly phosphorylated phosphophoryn (HP) binds a maximum of 1.33 calcium ions or 1.07 magnesium ions per organic phosphate residue at pH 7.4-8.0. The Ca-HP complexes are predominantly linear when the calcium content of the complex is less than about 65% of the saturation level. At higher calcium levels, the protein has a folded conformation, and transient protein-protein interactions occur. The equilibrium mixture of monomers and oligomers is predominantly monomeric unless the protein is saturated with calcium. The saturated Ca-HP complex forms discrete high molecular weight particles about 25 nm in diameter. The particles are electrically neutral and generally occur in clusters. Mg-HP complexes appear predominantly linear by electron microscopy at all concentrations of bound magnesium up to about 99% of the saturation level; however, protein-protein interaction is measurable when the magnesium content is as little as 65% of the saturation level. At saturation, Mg-HP complexes form high molecular weight particles which are negatively charged. Because of the negative charge, these particles form a stable colloidal suspension and have a rather stellate configuration.

High-capacity calcium-binding proteins are associated with calcium or mineral ion metabolism in extracellular or intravesicular environments where the free calcium concentration is in the millimolar range. In some tissues the function of the protein is obvious or interpretable from theoretical considerations. For example, the colloidal aggregates of casein and calcium phosphate in milk provide the fluid with a high content of suspended mineral ions (Schmidt, 1982). In muscle cells, calsequestrin is localized in the terminal cisternae of the sarcoplasmic reticulum and is thought to act as a calcium buffer by maintaining a low free calcium ion concentration (1-2 mM) and providing a large pool of readily dissociable bound calcium (20-30 mM) (MacLennan et al., 1983). In other tissues the function of the high-capacity calcium-binding proteins is unclear. The egg yolk protein phosvitin binds many calcium ions in vitro due to its high content of phosphoserine residues (Grizzuti & Perlmann, 1973), and presumably, a majority of the yolk calcium is associated with phosvitin in vivo. Dentin and bivalve phosphoproteins localized at sites of calcium phosphate and calcium carbonate deposition, respectively, have an undefined role in skeletal mineralization (Rahima et al., 1988; Marsh, 1986a).

High-capacity calcium-binding proteins self-associate in the presence of calcium ions. Native casein micelles are nearly spherical particles of variable size composed of submicelles ranging from 8 to 20 nm. Schmidt (1982) postulates that the subunits are linked together by $Ca_9(PO_4)_6$ clusters which interact with calcium phosphoserine complexes on adjacent submicelles. Consistent with this model, dephosphorylated casein does not aggregate in the presence of colloidal calcium phosphate (Aoki et al., 1987). In vitro calsequestrin crystallizes in the presence of 0.5-2.0 mM $CaCl_2$ (Maurer et al., 1985), and five different crystal forms have been identified (Williams & Beeler, 1986). In vivo calsequestrin forms an aggregated network in the terminal cisternae of the sarcoplasmic reticulum (Franzini-Armstrong et al., 1987) which appears paracrystalline in some preparations (Saito et al., 1984). In vivo the bivalve phosphoprotein occurs as discrete 40-nm particles which have a rather angular contour (Marsh & Sass, 1984). The integrity of the bivalve phosphoprotein particles is maintained by both calcium ions and covalent interchain cross-links through histidinoalanine residues (Sass

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