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Involvement of the carboxyl-terminal domain of tubulin in the regulation of its assembly

(limited proteolysis/protein/interactions/microtubules)

LUIS SERRANO, JAVIER DE LA TORRE, RICARDO B. MACCIONI, AND JESÚS AVILA

Centro de Biología Molecular (CSIC-UAM), Universidad Autónoma, Canto Blanco, Madrid-34, Spain

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ABSTRACT **Limited proteolysis of phosphocellulose-purified tubulin with subtilisin resulted in cleavage of both α and β tubulin subunits, with the formation of two major fragments (S_α, and S_β, 48 kDa) and a small peptide (4 kDa) containing the carboxyl-terminal region of tubulin. Interestingly, tubulin cleaved under the present conditions showed an increased ability to assemble into large polymers in the absence of MAPs and under conditions that do not promote assembly of undigested tubulin—i.e., low magnesium concentrations and the absence of taxol and polyalcohols. The critical concentrations for the subtilisin-cleaved tubulin assembly was similar to that of MAPs-promoted tubulin assembly. Assembly product from subtilisin-cleaved tubulin consisted mainly of protofilament bundles, hooked polymer, and open tubules, structures showing equatorial and longitudinal spacings of 50 and 40 Å, respectively. The existence of junctions between polymer walls indicates that the carboxyl-terminal removal facilitates polymer–polymer interactions. These results, together with previous studies on the involvement of the carboxyl-terminal domain of tubulin in its interaction with MAP-2, suggest a regulatory role for this domain in tubulin assembly. Thus, in general terms the tubulin molecule can be analyzed as a protein containing two essential domains with functional significance, one domain playing a major role in self-association and the other (the carboxyl-terminal moiety) playing a regulatory role in modulating the interactions responsible for self-association.**

Brain tubulin prepared by cycles of assembly–disassembly contains other proteins that are designated microtubule-associated proteins (MAPs). Tubulin stripped of MAPs is unable to assemble except under certain conditions far removed from the physiological ones. Such conditions involve solvents containing glycerol, dimethyl sulfoxide, or polyethylene glycol, and usually the presence of high concentrations of magnesium (5–10 mM) or the addition of taxol or polycations, which may substitute for the requirements of MAPs (e.g., see ref. 1). Recently it has been shown that the binding of one of these proteins to tubulin is through a cationic domain of MAP-2 (2), which binds to the anionic domain present in the carboxyl terminus of the α and β polypeptides of the tubulin molecule (3, 4). In this report we show that the removal of the carboxyl-terminal portion of the purified tubulin by limited proteolysis with subtilisin results in a molecule that can self-assemble in the absence of MAPs or taxol and at protein concentrations similar to those required for undigested tubulin in the presence of MAPs. The resulting polymeric structures are mainly hooked microtubules, folded sheets, and protofilament bundles.

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MATERIAL AND METHODS

Purification of Tubulin. Tubulin from pig brain was prepared by temperature-dependent cycles of assembly–disassembly by the procedure of Shelanski *et al.* (5) and was stored as pellets at −70°C. Immediately before use, the pellets were resuspended in 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (Mes), pH 6.4/0.5 mM $MgCl_2$/2.0 mM EGTA (buffer A), and a third cycle of assembly–disassembly was performed. Tubulin depleted of MAPs was obtained by phosphocellulose chromatography (PC-tubulin) as described by Weingarten *et al.* (6). The protein concentration of tubulin was determined by A_{280} = 1.15 mg/ml (7).

Proteolytic Digestions. Controlled proteolysis of PC-tubulin (2 mg/ml) with subtilisin (Sigma) was performed by incubation at 30°C for 30 min with 1% (wt/wt) subtilisin, unless otherwise indicated. Aliquots from the incubation mixture were obtained; after the addition of 1 mM phenylmethylsulfonyl fluoride and appropriate dilutions, the samples were assayed for assembly activity or treated for electrophoresis in the $NaDodSO_4$/polyacrylamide system.

Peptide maps of α- and β-tubulin subunits or the subtilisin-cleaved tubulin subunits (S_α and S_β) were obtained by digestion with formic acid, followed by polyacrylamide gel electrophoresis. The gel pieces containing either the tubulin subunits or the fragments S_α and S_β were incubated with 75% formic acid for 24 hr at 37°C (8). The acid was removed by lyophylization, and the gel pieces containing the digested protein were subjected to a polyacrylamide slab gel electrophoresis to separate the peptides produced in the digestion.

Assembly Assay. Undigested tubulin, subtilisin-digested tubulin, and microtubule protein samples were assembled by incubation at 37°C for 30 min in buffer A with 1 mM GTP. The assembled protein was isolated after centrifugation for 5 min at 150,000 × *g* in a Beckman Airfuge at room temperature. The protein present in the sedimented pellets was quantified by the method of Lowry *et al.* (9). Blanks were samples assayed under identical conditions except for the omission of GTP.

Crosslinking Reactions. PC-tubulin samples were digested with 1% subtilisin for 30 min at 30°C, and the reaction was stopped by adding 1 mM phenylmethylsulfonyl fluoride. Formaldehyde was added to a final concentration of 1% (wt/wt), and the mixture was incubated for 60 min at 4°C. The crosslinking reaction was terminated by addition of a 2-fold molar excess of glycine with respect to formaldehyde, and samples were subjected to electrophoresis.

Electrophoresis. Tubulin samples obtained during the proteolysis experiments or after crosslinking reactions were adjusted to 1% $NaDodSO_4$/1 M glycerol/2% (vol/vol) 2-mercaptoethanol/0.001% bromophenol blue, boiled, and

Abbreviations: MAPs, microtubule-associated proteins; Mes, 2-(*N*-morpholino)ethanesulfonic acid; PC-tubulin, tubulin purified by phosphocellulose chromatography.

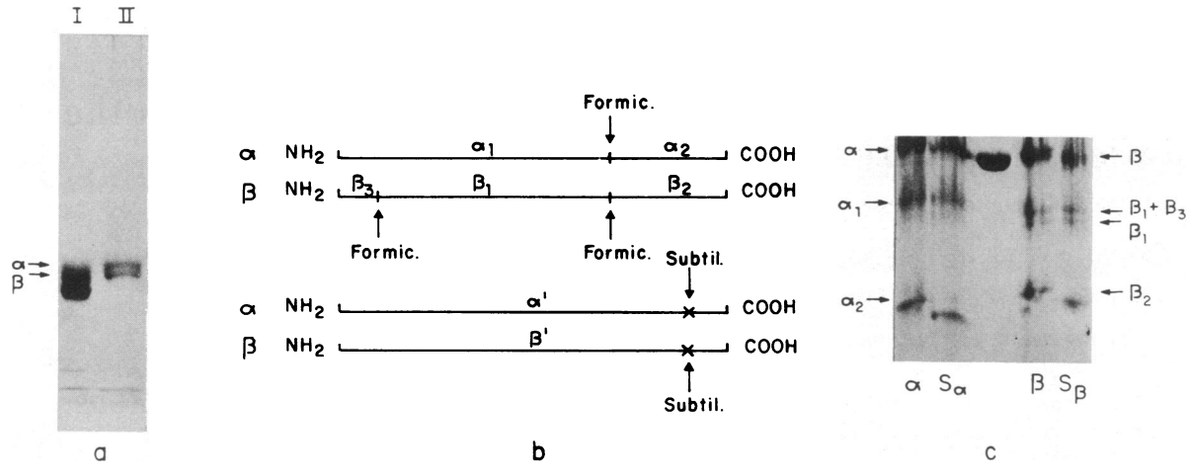

FIG. 1. Removal of carboxyl-terminal fragments of tubulin subunits by subtilisin. (*a*) PC-tubulin (2 mg/ml; 0.1 ml) was incubated with 1% subtilisin for 30 min at 30°C in buffer A. After proteolysis with subtilisin, the peptides were fractionated by electrophoresis in 7.5% polyacrylamide gels, and the patterns of digested (lane I) and undigested (lane II) tubulin were compared. (*b*) Schematic representation of the formic acid cleavage of both α- and β-tubulin subunits. The subtilisin limited cleavage of α and β tubulin is also shown. (*c*) Formic acid peptide maps, indicating the migration of fragments obtained from either α- or β-tubulin subunits, and the peptide maps of S_α and S_β. Electrophoresis was run in 12.5% acrylamide gels.

analyzed by electrophoresis in $NaDodSO_4$/polyacrylamide slab gels (7–12.5%) by the procedure of Laemmli (10) with a constant current of 20 mA per gel. Gels were stained by the method of Fairbanks *et al.* (11) with Coomassie blue.

Electron Microscopy. The assembly products of either undigested or subtilisin-digested tubulin samples were analyzed in thin sections roughly perpendicular to the polymers, as described by Mandelkow and Mandelkow (12). Furthermore, assembled samples were placed on carbon/collodion-coated grids and stained with 1% (wt/wt) uranyl acetate as described and observed in a Jeol 100B electron microscope (13).

RESULTS AND DISCUSSION

Previous results (3, 4) on the partial proteolysis of tubulin with subtilisin have indicated the cleavage of tubulin at two sites: one cleavage resulted in fragments of 32 and 20 kDa and the other produced a 48-kDa fragment as well as a small peptide (4 kDa) containing the carboxyl-terminal region of tubulin subunits. Limited proteolysis of PC-tubulin with subtilisin under milder conditions (1% subtilisin) than was used in the previous studies resulted in formation of two major polypeptides (S_α and S_β, 48–49 kDa) with a faster electrophoretic mobility in the $NaDodSO_4$/polyacrylamide system than that of the intact tubulin subunits (Fig. 1*a*), indicating the removal of a small fragment (4 kDa) from both α and β subunits. This small fragment corresponds to the carboxyl-terminal segment of the tubulin subunits as confirmed here by formic acid peptide mapping (Fig. 1*b*). The tubulin sequence described by Ponstingl *et al.* (14) showed the presence of an aspartic acid-proline bond at the positions 306–307 in the α-tubulin subunit and at the positions 61–62 and 306–307 in the β-tubulin subunit. Since aspartic acid-proline bonds are cleaved by incubation with formic acid (8), we have obtained formic acid peptide maps of subtilisin-digested and undigested tubulin subunits. This peptide mapping showed a faster electrophoretic mobility of the carboxyl-terminal fragments of subtilisin-digested tubulin subunits (S_α and S_β) as compared with those of undigested tubulin α and β subunits (Fig. 1*c*). Electrophoretic analysis after partial digestion of microtubule protein showed a higher extent of proteolysis of β tubulin, suggesting that MAPs may protect preferentially the α subunit against cleavage (Fig. 2). A densitometric tracing of the gel showed that, after subtilisin limited proteolysis of microtubular protein, the amount of α-tubulin subunit was 42.1% of the protein in the gel, while the remaining β subunit was only 12.2%, the rest being proteolytic fragments (Fig. 2, lane B).

Tubulin subunits lacking their carboxyl-terminal segment (S_α and S_β) interacted, as evidenced by formation of a subtilisin-cleaved tubulin dimer and higher order oligomers after formaldehyde crosslinking. The crosslinked dimer of subtilisin-digested tubulin exhibited an electrophoretic mobility faster than that of the crosslinked dimer of intact tubulin (Fig. 3*a*). Our previous studies (3, 4) have indicated that limited proteolysis of microtubular protein (2% subtilisin for 30 min) results in a product that exhibits decreased microtubule assembly as compared with the high extent of assembly of the undigested control. A partial decay of assembly ability of subtilisin-digested tubulin also has been observed when assembly was assayed in the presence of taxol under conditions where the undigested control presented a maximal assembly activity (4). The self-assembly of tubulin or subtilisin-cleaved tubulin in the absence of MAPs

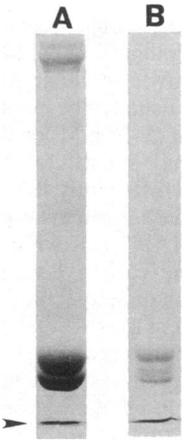

FIG. 2. Microtubule protein (2 mg/ml; 0.1 ml) was incubated with 1% subtilisin for 30 min at 30°C. The reaction was stopped with 1 mM phenylmethylsulfonyl fluoride, and the samples were subjected to electrophoresis in a polyacrylamide slab gel (7.5% acrylamide). Lanes: A, undigested microtubule protein; B, digested microtubule protein.

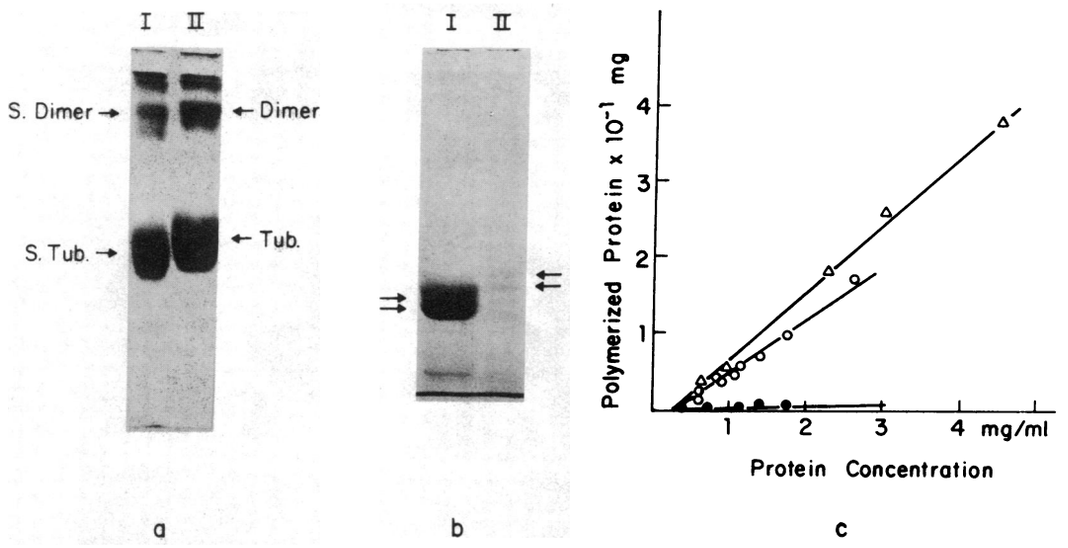

FIG. 3. Crosslinking of subtilisin-digested tubulin. (*a*) PC-tubulin (2 mg/ml; 0.1 ml) was digested with 1% subtilisin for 30 min at 30°C. The reaction was stopped by adding 1 mM phenylmethylsulfonyl fluoride. Formaldehyde was added (final concentration, 1%), and the mixture was incubated for 60 min at 4°C. The reaction was stopped by the addition of glycine/formaldehyde, 2:1 (mol/mol). Crosslinked peptides from subtilisin-digested tubulin (S. Tub.) (lane I) and undigested tubulin (Tub.) (lane II) were fractionated by electrophoresis. (*b*) Subtilisin-digested PC-tubulin (2 mg/ml; 0.1 ml) in buffer A plus 1 mM GTP was incubated for 30 min at 35°C. After incubation, polymerized protein was isolated by centrifugation for 5 min at 150,000 × *g* in a Beckman Airfuge. Protein characterization of the pelleted (lane I) and nonpelleted (lane II) fractions and the presence of α and β tubulin in the supernatant are indicated. (*c*) Subtilisin-digested PC-tubulin (0.15 ml) was polymerized at different protein concentrations under the conditions indicated in *b*. Polymerized protein was quantified after centrifugation, and the amount of such protein was plotted versus protein concentration present in the incubation mixture before assembly (○). A similar experiment was done with microtubule protein isolated by the method of Shelanski *et al.* (5) (△) and with undigested PC-tubulin (●). Digestion with 4% (wt/wt) subtilisin resulted in further cleavage of tubulin subunits, and no polymerization was found.

or taxol and under milder conditions of proteolysis was examined here. Interestingly, subtilisin-cleaved tubulin could assemble into large polymers in the absence of MAPs and under conditions that did not promote assembly of undigested PC-tubulin—i.e., low magnesium and low protein concentrations and the absence of taxol or polyalcohols. Tubulin cleavage followed by assembly and electrophoretic analysis of polymerized products showed that S_α and S_β tubulin fragments appeared in the polymer pellets, while a high proportion of α and β subunits remained in the supernatant (Fig. 3*b*). Since polymers of subtilisin-cleaved tubulin are sensitive to cold and can be subjected to cycles of assembly–disassembly, which procedure removes the subtilisin activity present in the incubation mixture (data not shown), it appears that this increase in the assembly ability is a consequence of the removal of the carboxyl-terminal fragment of tubulin subunits and not to the presence of subtilisin. The dependence of polymerization of subtilisin-cleaved tubulin on protein concentration indicates that the critical concentration (15) required for assembly was similar to that of tubulin in the presence of MAPs (Fig. 3*c*). Under the present experimental conditions, no significant assembly was obtained from the pure undigested tubulin.

Characterization of the polymers of subtilisin-cleaved tubulin by electron microscopy indicated the presence of structures resembling partially open microtubules, folded sheets, and protofilament bundles (Fig. 4 *a* and *b*). Optical diffraction of micrographs of open tubules from subtilisin-cleaved tubulin showed the existence of an equatorial spacing of 50 Å for these structures and a longitudinal spacing of 40 Å, similar to those described for microtubules (17) (Fig. 4*c*). Thin sections roughly perpendicular to the polymer axes showed the existence of hooked microtubules (Fig. 4*a*) similar to those assembled *in vitro* under certain conditions (18) or those described in a *Drosophila hydei* mutant (19). Such polymers also resembled natural structures such as flagellar doublets or centrioles. The structures in Fig. 4*a* showed the existence of junctions between polymer walls, indicating an increased number of lateral (interprotofilament) interactions, as compared with those of microtubules. It suggests that lateral interactions are facilitated upon removal of the carboxyl-terminal region of tubulin subunits. The results show that molecules of subtilisin-cleaved tubulin have increased ability to self-associate, thereby assembling into polymeric structures that also exhibit polymer–polymer interactions. On the other hand, association of subtilisin-cleaved tubulin mainly into tubules, hooked polymers, or protofilament bundles suggests that the carboxyl-terminal domain normally functions in stabilizing multimeric arrays of tubulin, forming separate microtubules. Noteworthy is the high α helix potential of the carboxyl-terminal moiety of tubulin, which could contribute to creation of an appropriate tubulin conformation, thus controlling the levels of interaction with other tubulin molecules. Furthermore, it is likely that MAPs provide the optimal conditions for microtubule assembly by charge neutralization (3, 4) and conformational stabilization of tubulin.

These findings along with previous studies (3, 4) suggest a regulatory function for the carboxyl-terminal region of tubulin subunits in the mechanism of tubulin assembly. This regulation could operate through two opposing mechanisms: (*i*) favoring the tubulin–tubulin interactions by electrostatic neutralization of the acidic domain by MAPs, as in the case of the enzymatic removal of the carboxyl-terminal domain from tubulin, and (*ii*) hindering the interactions that might occur in the absence of MAPs and, thus, modulating the assembly into microtubule structures. Thus, the tubulin molecule can be analyzed as a protein composed of two major domains: one large domain intimately involved in the interactions with other tubulin subunits and the other (the carboxyl-terminal moiety) playing a regulatory role in modulating the interactions responsible for self-association. Structural domains in the tubulin molecule playing functional roles, such as those for interaction with nucleotides or the colchicine-binding site, have been studied (20–23) and appear to be topographically located within the larger region

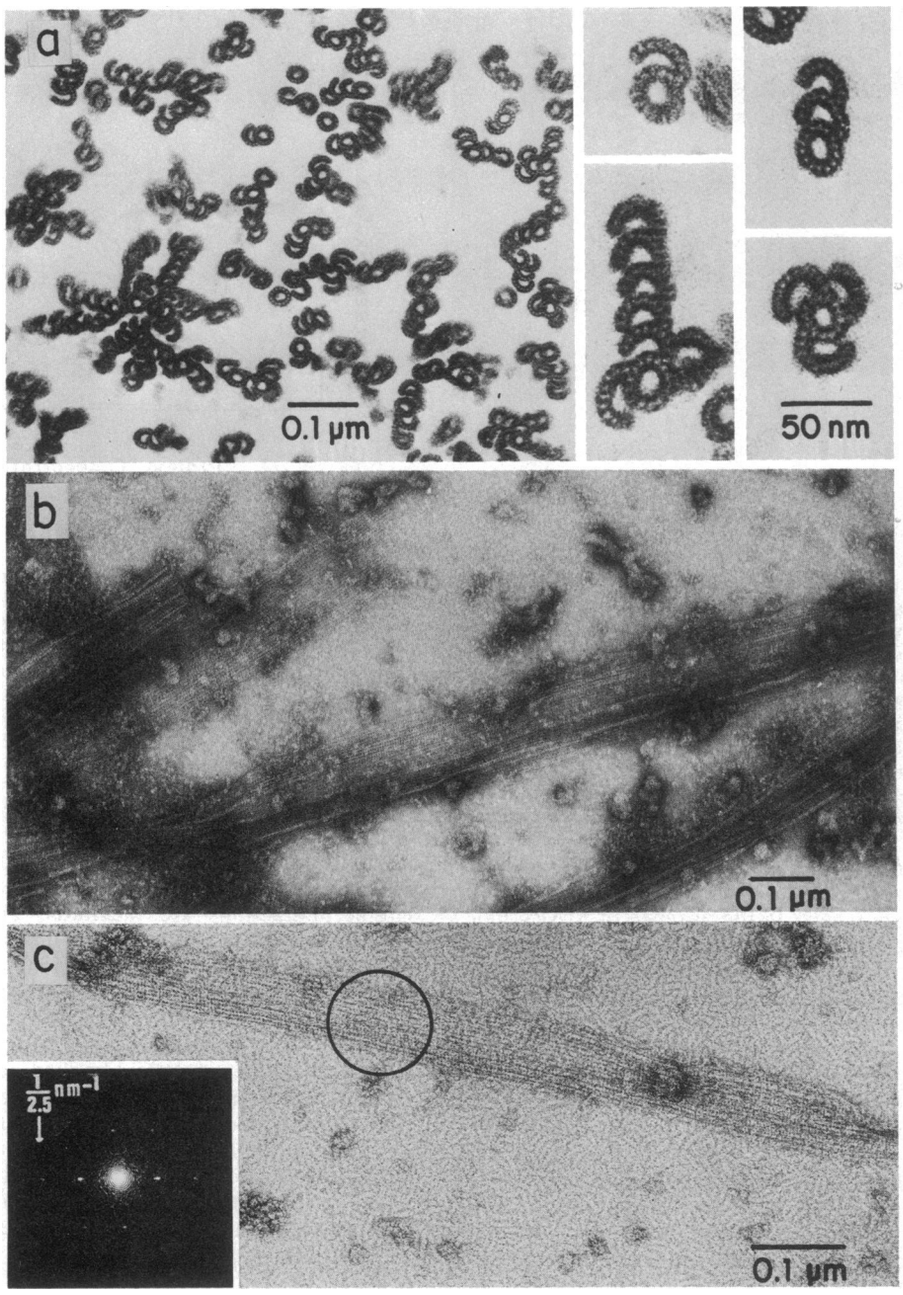

FIG. 4. Characterization of subtilisin-digested tubulin polymers. (*a*) Subtilisin-digested PC-tubulin was polymerized as indicated in Fig. 3*b*, and thin sections roughly perpendicular to subtilisin-digested polymers were obtained as indicated by Mandelkow and Mandelkow (12). A general view and some details are shown. (*b*) Samples of the incubation mixture indicated in *a* were placed on a carbon/collodion-coated grid and stained with 1% uranyl acetate as indicated. The grids were observed in a Jeol 100B electron microscope. Similar results were obtained with the mica flotation technique. This technique has been used for tubulin sheets containing MAPs (16) but was unsuitable for MAPs-free sheets because of the interaction of an anionic tubulin domain (perhaps the carboxyl-terminal domain) with the mica support. (*c*) Several micrographs were screened by optical diffraction (13), and the pattern obtained for one of them is indicated (circle). Optical diffraction of catalase crystals in the same grids was done as an internal control of dimensions.

corresponding to the 48-kDa segment of either α- or β-tubulin subunits.

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