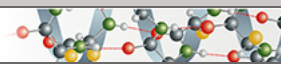


**Protein Structure and Folding:
Roles of β -Tubulin Residues Ala428 and
Thr429 in Microtubule Formation in Vivo**

PROTEIN STRUCTURE
AND FOLDING



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Roles of β -Tubulin Residues Ala⁴²⁸ and Thr⁴²⁹ in Microtubule Formation *in Vivo**

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The C termini of β -tubulin isotypes are regions of high sequence variability that bind to microtubule-associated proteins and motors and undergo various post-translational modifications such as polyglutamylation and polyglycylation. Crystallographic analyses have been unsuccessful in resolving tubulin C termini. Here, we used a stepwise approach to study the role of this region in microtubule assembly. We generated a series of truncation mutants of human β I and β III tubulin. Transient transfection of HeLa cells with the mutants shows that mutants with deletions of up to 22 residues from β III and 16 from β I can assemble normally. Interestingly, removal of the next residue (Ala⁴²⁸) results in a complete loss of microtubule formation without affecting dimer formation. C-terminal tail switching of human β I and β III tubulin suggests that C-terminal tails are functionally equivalent. In short, residues outside of 1–429 of human β -tubulins make no contribution to microtubule assembly. Ala⁴²⁸, in the C-terminal sequence motif N-QQYQDA⁴²⁸, lies at the end of helix H12 of β -tubulin. We hypothesize that this residue is important for maintaining helix H12 structure. Deletion of Ala⁴²⁸ may lead to unwinding of helix H12, resulting in tubulin dimers incapable of assembly. Thr⁴²⁹ plays a more complex role. In the β I isotype of tubulin, Thr⁴²⁹ is not at all necessary for assembly; however, in the β III isotype, its presence strongly favors assembly. This result is consistent with a likely more complex function of β III as well as with the observation that evolutionary conservation is total for Ala⁴²⁸ and frequent for Thr⁴²⁹.

Microtubules are involved in a great variety of cellular functions. Their constituent protein tubulin is an $\alpha\beta$ heterodimer, both α - and β -tubulin existing as multiple isotypes, encoded by different genes and differing in amino acid sequence (1). The differences among the isotypes are highly conserved in evolution. In mammals, the β isotypes are β Ia, β Ib, β II, β III, β IVa, β IVb, β V, and β VI. There is evidence that the isotype differences have functional significance. For instance, the β IV isotype is found in all axonemes (2).

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Structurally, both α - and β -tubulin consist of a globular region of 427 amino acids followed by a C-terminal region of 17–24 amino acids (3–5). The C-terminal region is highly negatively charged, being especially rich in glutamate residues and lacking in basic residues, and is likely to project outward from the rest of the molecule, because of its high negative charge and the electrostatic repulsion among the glutamate residues (3). The three-dimensional structure of the globular domain has been determined by electron and x-ray crystallography (4, 5). However, the C-terminal region has never been localized in the three-dimensional reconstructions except by computer modeling. The probable reasons for this are 1) that, if the C-terminal region projects out from the rest of molecule, it is likely to be very flexible with respect to the rest of the molecule and 2) the C-terminal region undergoes post-translational modification. Both of these can lead to structural heterogeneity and cause the C terminus to be invisible to crystallographic techniques.

In this work, we examine the role of the C termini of human β -tubulins to determine the minimal sequence requirement for microtubule incorporation through structure/function analyses. The human β I and β III tubulin isotypes were utilized based on their high degree of sequence variability clustered at the C terminus (Table 1 and Fig. 1) and the fact that β I is broadly distributed among normal tissues, whereas β III has a very narrow tissue distribution. These two isotypes share 92% sequence identity, with differences among these isotypes occurring in both the globular domain and the C-terminal region (1).

Three attributes of potential functional significance have been assigned to the C-terminal regions of tubulin. First, the fact that it projects outward makes it likely that it can serve as a signal. For example, elegant experiments by Popodi *et al.* (6), working with β -tubulin isotypes from *Drosophila*, indicate that the C terminus is the region that determines which isotype goes into axonemal microtubules. In *Tetrahymena thermophila*, Duan and Gorovsky (7) demonstrated that α - and β -tubulin C-terminal tails (CTT)² are interchangeable, and their functions are indistinguishable. In addition, a duplicated α -tubulin CTT rescued the lethal mutant lacking post-translational modification sites on β -tubulin but did not rescue the mutant lacking a 17-amino acid deletion from the β -tubulin tail (7). A significant amount of research on C-terminal tail function has utilized proteolytic digestion with a number of different endoproteases such as subtilisin, proteinase K, and chymotrypsin among others (8–10). For example, subtilisin-digested $\alpha_s\beta_s$ -tubulin was found to have a higher capacity for generating

² The abbreviations used are: CTT, C-terminal tail; MAP, microtubule-associated protein; FL, full length; PBS, phosphate-buffered saline.

TABLE 1
The C-terminal amino acid sequences of the human β -tubulin isotypes

Human β -tubulin isotype	C-terminal tail sequence
β Ia	QQYQDATAEEEEEDFGEEAEAAA
β Ib	QQYQDATAEEEEEDFGEEAEAAA
β II	QQYQDATADEQGEFEEEEGEDEA
β III	QQYQDATAEEEGEMYEDDEESEAQGP
β IVa	QQYQDATAEQGEFEEEEAEVVA
β IVb	QQYQDATAEEEGEFEEEEAEVVA
β V	QQYQDATAANDGEEAFEDDEEEDIG
β VI	QQFQDAKAVLEEDDEVTEEAEMEPEDKGH
β VII	QQYQDATAEGEGV
β Ia	MREIVHIQAGQCGNQIGAKFWEVISDEHGIDPTGTGYHGDSDLQLDRISVY 50
β III	-----S-N-V-----E----- 50
β Ia	YNEATGGKYVPRAILVDLEPGTMDSVRSRGGPFQIFRPDNFVFGQSGAGNN 100
β III	----SSH-----A--HL-----I----- 100
β Ia	WAKGHYTEGAELVDSVLDVVRKEAESCDCLQGFQLTHSLGGGTGSGMGTL 150
β III	-----C-N----- 150
β Ia	LISKIREEYPDRIMNTFSVVPSPKVSDTVVEPYNATLSVHQLVENTDETY 200
β III	----V-----I----- 200
β Ia	CIDNEALYDICFRTLRLTPTYGDLNLHVSGMTTCVTCRFPFQQLNADL 250
β III	-----K-A-----A--SG--S----- 250
β Ia	RKLAVNMVFPRLHFFMPGFAPLTSRGSQQYRALTVPDLTQQVFDANKMM 300
β III	-----A-----E--M----- 300
β Ia	AACDPRHGRYLTVAAVFRGRMSKVEDEQMLNVQNKNSYFVEWIPNNVK 350
β III	-----T-----AI-S----- 350
β Ia	TAVCDIPRGLKMAVTFIGNSTAIQELFKRISEQFTAMFRRKAFLHWYTG 400
β III	V-----SS----- 400
β Ia	EGMDEMEFTEAESNMNDLVSEYQQYQDATAEEEEEDFGEEAEAAA 444
β III	-----GEMY-DD---SEAQGP 450

FIGURE 1. Sequence alignment of human β Ia and β III tubulin isotypes. Human β Ia and β III tubulin isotypes were aligned with ClustalX 1.83 and processed with BioEdit. Hyphens denote identical residues between sequences.

microtubules than undigested (9). A single drawback to using these proteases is their site-specific nature, which limits us to distinct digestion sites in proteolysis experiments. Furthermore, the proteolyzed tail fragment could still interact with the globular body without being really separated. Thus, to elucidate the importance of amino acids flanking these digestion sites, alternative approaches must be utilized.

Second, MAPs and motor proteins such as MAP2, MAP4, tau, DMAP-85, OP18/stathmin, dynein, and kinesin have been shown to bind the C-terminal region (11–22). These proteins are known to play very important roles in cellular processes including intracellular transport and modulation of microtubule dynamics. Third, the C terminus is subject to a large number of post-translational modifications, some of which are known to have functional significance (1). These include phosphorylation (β) (23–25), polyglutamylation (α , β) (26–30), polyglycylation (α , β) (31–34), detyrosination (α) (35–37), and deglutamylation (α) (38).

In this paper we present evidence for a fourth function for the C-terminal region, namely, that it plays a major role in controlling the conformation of the globular region of the tubulin molecule such that microtubules can form. We have found that all of the amino acid residues necessary for assembly of the β I isotype of tubulin are contained within the first 428 amino

acids, ending in N-QQYQDA⁴²⁸; C-terminal truncations lacking Ala⁴²⁸ yield tubulins that are not compatible with microtubule formation. We demonstrate that the C-terminal region does not contribute to intradimer formation. Furthermore, we find that β -tubulin C-terminal tail switching does not affect incorporation and that the presence of the full chimeric tail is not necessary for functional microtubules. Finally, we have observed that residue Thr⁴²⁹ plays an important but not critical role in the β III isotype becoming assembly-competent but is not at all necessary for the β I isotype to form microtubules.

EXPERIMENTAL PROCEDURES

Materials—HeLa human cervical carcinoma cells were purchased from the American Type Culture Collection (Manassas, VA). Fetal bovine serum was purchased from Atlanta Biologicals (Lawrenceville, GA). Dulbecco's modified Eagle's medium, Hanks' balanced salt solution, trypsin-EDTA, TOPO cloning kit, pcDNA3.1/V5-His-TOPO vector, anti-V5 antibody (Mo), anti-glyceraldehyde-3-phosphate dehydrogenase (Mo), and 4',6-diamidino-2-phenylindole were purchased from Invitrogen. Penicillin-streptomycin-fungisone antibiotics were from Gemini Bio-Products (West Sacramento, CA). Vectashield mounting medium was from Vector Laboratories (Burlingame, CA). G-418 sulfate was purchased from American Bioanalytical (Natick, MA). The Super Signal kit was from Pierce. Normal goat serum, Cy3-conjugated goat anti-mouse antibody, horseradish peroxidase-conjugated anti-mouse antibody were purchased from Jackson ImmunoResearch (West Grove, PA). FuGENE HD transfection reagent was from Roche Applied Science. Taq PCR Master Mix kit was purchased from Qiagen.

Molecular Constructs—Polymerase chain reaction was used to create chimeras and C-terminal truncations for human β I and β III tubulin (GenBankTM accession numbers AF427491 and NM_178014, respectively) (Table 2) with the appropriate forward and reverse primers at final concentrations of 1 μ M each (Table 3). Amino acid deletions made to the β III C-terminal end were the following: Δ 5, Δ 10, Δ 20, Δ 21, Δ 22, Δ 23, Δ 24, Δ 25, and Δ 30. Wild type β III tubulin was also constructed as a positive control. For β I tubulin, C-terminal deletions resulted in Δ 16 and Δ 17 constructs. Taq PCR Master Mix kit and cycling parameters were performed according to Qiagen. Two rounds of PCR were required to produce β III+C β I tubulin consisting of the first 433 amino acids from β III tubulin and the last 11 amino acids from β I tubulin. The first round produced β III+C β I C Δ 5, which contained a five-amino acid deletion from the β I C-terminal tail, whereas the second round generated the full-length β I tail (Table 2). The reciprocal chimera β I+C β III, containing the first 433 amino acids from β I tubulin and the last 17 amino acids from β III tubulin, required four successive PCRs that generated β I+C β III C Δ 13, β I+C β III C Δ 9, β I+C β III C Δ 5 lacking the last 13, 9, and 5 amino acids, respectively, from the β III tubulin tail (Table 2). The last of four rounds of PCR yielded the full-length chimera (Table 2). After each round of PCR, the product was diluted to final concentrations of 1:100 and 1:1000 dilutions prior to subsequent PCR. All of the constructs were TA cloned into pcDNA3.1/V5-His-TOPO and tagged with a C-terminal V5-His tag according to the manufacturer's protocol (Invitrogen). Addi-

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sequence alignment, hyphens represents identical residues between sequences (Fig. 1).

Immunofluorescence Microscopy—All of the cells were grown on glass coverslips at 37 °C and 5% CO₂. The cells were washed once with Hanks' balanced salt solution, fixed for 15 min with 3.7% paraformaldehyde at room temperature, and permeabilized for 1 min with 0.5% Triton X-100 in PBS (0.15 M NaCl, 0.0027 M KCl, 0.00147 M KH₂PO₄, 0.01 M Na₂HPO₄, pH 7.2). The cells were then incubated overnight at 4 °C with the anti-V5 antibody (1:1000) diluted in PBS containing 10% normal goat serum. For HeLa cells transfected with β I+C β III (without V5-His tag), the cells were stained with the monoclonal antibody to β III tubulin (SDL3D10) (1:500) diluted in PBS containing 10% normal goat serum. The cells were rinsed in PBS and labeled with Cy3-conjugated goat anti-mouse antibody (1:100) for 1 h at room temperature. The cells were then rinsed three times with PBS. For DNA detection, the cells were stained with 4',6-diamidino-2-phenylindole (7.15 μ M in PBS) for 5 min at room temperature followed by three washes with PBS. The coverslips were mounted on glass slides with Vectashield mounting medium and examined with a Olympus BX60 epifluorescence microscope coupled to a Spot camera (model 1.4.0 from Diagnostic Instruments, Inc.) using an Olympus PlanApo 60 \times oil immersion objective. For each image, a single cell was chosen for 2 \times digital expansion (120 \times total magnification) to view cortical microtubules. All of the images were processed using Adobe Photoshop 7.0.

Quantitation of Microtubule-forming Cells—HeLa cells transfected with wild type, C-terminal truncated mutants, and chimeras of β I and β III were subjected to analysis and quantitation of microtubule formation in four random fields by indirect immunofluorescence microscopy as described under "Experimental Procedures." In each field, the transfected, microtubule-forming cells were counted and compared with the total number of transfected cells (both microtubule-forming and non-microtubule-forming cells) expressed as a percentage. For each cell line, the fields were averaged, and standard deviations are given (see Fig. 5).

Co-immunoprecipitation of C-terminally Truncated β I and β III Tubulin—Extracts from transfected HeLa cells were prepared with cell lysis buffer (50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 1% Nonidet P-40, 1% protease inhibitor mixture, 2 mM phenylmethylsulfonyl fluoride) according to Banerjee (41). Supernatant was cleared with 200 μ l of protein G-agarose beads (50% slurry) for 1 h at 4 °C. Cleared supernatant was subsequently incubated with 3 μ l of anti-V5 antibody overnight at 4 °C. The immunoprecipitated complex was captured with 50 μ l of protein G-agarose (50% slurry) at 4 °C for 1 h and washed twice in cell lysis buffer. The immunocomplex was resuspended in SDS-PAGE sample buffer and heated at 85 °C for 10 min. Precipitated protein was subjected to SDS-PAGE and Western analysis similar to that of Banerjee (42) utilizing anti- α -tubulin (1:5000) antibody and horseradish peroxidase-conjugated anti-mouse (1:1000) to probe the nitrocellulose membrane.

Western Blot Analysis—Cell extract preparation was done as described by Banerjee (41), followed by measurement of total protein concentration as described by Lowry *et al.* (43). Approximately 50 μ g of total cellular protein from HeLa cells

expressing β I constructs and 30 μ g of total cellular protein from HeLa cells expressing β III or chimeric β -tubulin constructs was loaded into a SDS-PAGE gel (8–16% linear gradient) and run in a manner similar to that of Banerjee (41). Immunoblotting was performed by methods described by Banerjee (42). The blots were incubated with anti-V5 antibody (1:5000). The secondary horseradish peroxidase-conjugated anti-mouse antibody (1:5000) was utilized with the Pierce Super Signal Kit to analyze expression of the transfected cell lines through chemiluminescence. The blots were reprobed with anti-glyceraldehyde-3-phosphate dehydrogenase as a loading control.

RESULTS

***In Vivo* Analysis of C-terminal Truncations of Human β I and β III Tubulin**—Deletions were made to human β I and β III tubulin at the C-terminal end to identify the minimum sequence and number of residues required for microtubule assembly *in vivo*. We first addressed the question of whether it was preferable to add a reporter group to the N-terminal or the C-terminal end of β III-tubulin. Preliminary experiments showed that constructs of wild type β III tubulin tagged with an N-terminal green fluorescent protein or hemagglutinin were incapable of forming microtubules as visualized by immunofluorescence (data not shown). The fact that both large and small N-terminal tags (green fluorescent protein and hemagglutinin, respectively) interfered with wild type β III incorporation is not surprising, considering the amount of conservation in this region. Across all species, there is 52 and 40% conservation in intra- and inter-dimer interface residues (44). Introducing bulky groups to this region could very well interrupt the loop T1 hydrogen-bonding interactions with the adjacent nucleotide at the interdimer interface. As a result of these experiments, we decided to add the reporter group to the C terminus of β -tubulin; constructs were created with a C-terminal V5-His tag to distinguish the transfected mutant β -tubulins from endogenous β -tubulins. Nine C-terminal truncations of varying length were made to β III tubulin and transfected into HeLa cervical carcinoma cells along with β III wild type as a positive control (Table 2). Of the ten, only β III wild type, β III C Δ 5, β III C Δ 10, β III C Δ 20, β III C Δ 21, and β III C Δ 22 were incorporated into interphase microtubules as viewed by immunofluorescence (Fig. 2*a*). The other constructs, namely β III C Δ 23, β III C Δ 24, β III C Δ 25, and β III C Δ 30, were not found in interphase microtubules after transfection (Fig. 2*a*). The inflection point demonstrating forming and non-microtubule-forming constructs was surprisingly abrupt, with the β III C Δ 22 construct showing significantly less incorporation into microtubules (Fig. 2*b*), whereas the β III C Δ 23 (Fig. 2*b*) and shorter constructs showed no incorporation (Fig. 2*a*). Constructs of β III that did not incorporate into microtubules appeared to form aggregates. These transfected cells lacked any form of microtubule architecture, and the truncated proteins were evenly distributed throughout the cell.

As a comparison with the β III constructs, we also examined the β I isotype. Various constructs of β I were created and transfected into HeLa cells. Similar results were obtained as with β III, except that it took much shorter truncations to produce similar results. However, because β III has a longer C terminus than does β I, this is not a surprising result. The transition

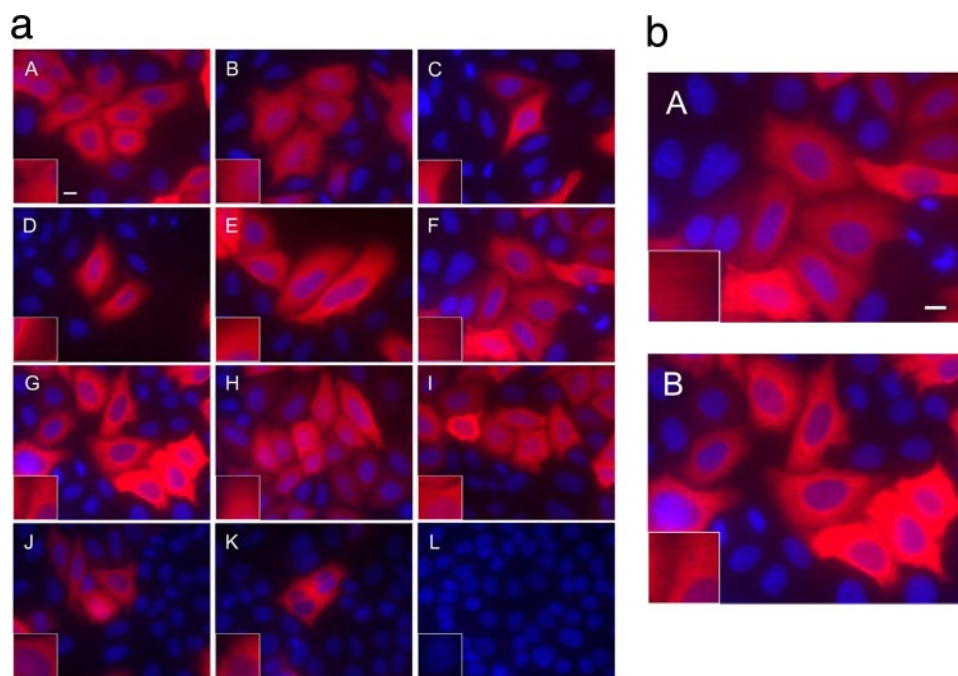


FIGURE 2. Microtubule incorporation of β III tubulin is abrogated with C-terminal tail truncations larger than 22-amino acids. Panel *a*, HeLa cells were transfected with β III wild type (A), β III C Δ 5 (B), β III C Δ 10 (C), β III C Δ 20 (D), β III C Δ 21 (E), β III C Δ 22 (F), β III C Δ 23 (G), β III C Δ 24 (H), β III C Δ 25 (I), and β III C Δ 30 (J). The positive control was expressing V5-His tagged β -galactosidase (K), whereas the negative control was of nontransfected HeLa cells (L). Twenty-four hours post-transfection, the cells were fixed and labeled with an antibody to V5 tag followed by Cy3-conjugated goat anti-mouse and visualized at 60 \times magnification. Microtubules are shown in red, and nuclei are stained blue. Insets, visualization of cortical microtubules after 2 \times digital expansion. Panel *b*, inflection point of microtubule and non-microtubule-forming cells (β III C Δ 22 (A) and β III C Δ 23 (B), respectively) from transfected HeLa culture. Bar, 10 μ m.

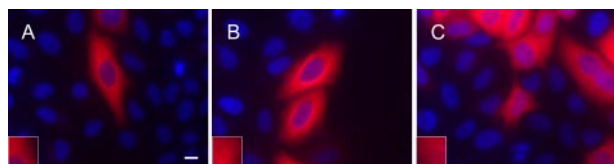


FIGURE 3. Microtubule incorporation of β I tubulin is abrogated with C-terminal tail truncations larger than 16 amino acids. Human β I wild type (A), β I C Δ 16 (B), and β I C Δ 17 (C) were transfected in HeLa cells. Twenty-four hours post-transfection, the cells were fixed and labeled with an antibody to V5 tag followed by Cy3-conjugated goat anti-mouse and visualized at 60 \times magnification. Insets, visualization of cortical microtubules after 2 \times digital expansion. Notice that none of the cells transfected with β I C Δ 17 (C) were able to form microtubules. Bar, 10 μ m.

between being able to form microtubules and not being able to form microtubules occurred at the corresponding point in the sequence for both β III and β I. Clearly, all the residues required for microtubule assembly are contained within the first 428 amino acids in both β I and β III. Interestingly, the transition between being able to incorporate into microtubules and not being able to do so was even more abrupt than was the case with β III, with β I C Δ 16 incorporating normally and β I C Δ 17 not at all (Fig. 3). As was the case with β III, the construct of β I that was unable to incorporate into microtubules appeared to form an aggregate instead (Fig. 3). Analysis through sequence alignment indicated that the first 428 amino acids of human β I and β III tubulin, ending with the amino acid sequence N-QQYQDA⁴²⁸, contained the residues required for proper insertion into microtubules (Fig. 1). Digital expansion of the cortical region of microtubules clearly confirms that deletions up to β I C Δ 16 and

β III C Δ 22 are assembly-competent, whereas deletions beyond are not (Figs. 2 and 3).

Chimeras of β I and β III Tubulin Do Not Require the Opposing, Full-length, C-terminal Tail for Microtubule Incorporation in Vivo—The previous results indicated that residues from 429 on, in β I- or β III-tubulin were not required for proper assembly into microtubules, but they did not address the question of whether altering the amino acid sequence of the C-terminal region could interfere with microtubule assembly. To address this point, chimeras of human β I and β III tubulin were constructed and transfected into HeLa cells to see whether combining the region 1–433 of one isotype with the C-terminal region of another would affect incorporation into microtubules (Table 2). CTT switching resulted in functional microtubules (Fig. 4). The six variations of chimeras created were β III+C β I FL (full length), β I+C β III FL¹, β I+C β III C Δ 5, β I+C β III C Δ 9, and β I+C β III C Δ 13 (Table 2). The

first of these consists of amino acids 1–433 of β III with the full-length C-terminal region of β I (the last 11 amino acids of β I). The second is the converse construct: 1–433 of β I with the full-length C-terminal region (last 17 amino acids) of β III. The other constructs consist of β I with variously truncated segments of the C terminus of β III. Our immunofluorescence results show that β III+C β I FL and β I+C β III FL are capable of entering microtubules (Fig. 4). Additionally, β I+C β III C Δ 5, β I+C β III C Δ 9, and β I+C β III C Δ 13, representing five-, nine-, and thirteen-amino acid deletions from the β III C-terminal tail, showed no significant difference from the full-length chimera (β I+C β III FL) (Fig. 4). As a control, a chimera of β I+C β III FL was created without a V5-His tag to show that the tag does not contribute or interfere with the ability to form microtubules (Fig. 4). We conclude that CTT switching between human β I and β III, after the first 433 amino acids, is not deleterious to microtubule formation.

The β III Tubulin Isoform Is Less Resistant to C-terminal Deletion than β I Tubulin—Quantitation of microtubule-forming cells as a percentage of the total transfected population was performed as described under “Experimental Procedures.” These data suggested that C-terminal deletions up to 21 amino acids were very well tolerated with 94.5 ± 3.1 , 91.2 ± 10.1 , 87.1 ± 11.0 , 80.2 ± 19.2 , and $82.2 \pm 3.2\%$ of transfected HeLa cells incorporating β III wild type, C Δ 5, C Δ 10, C Δ 15, C Δ 20, and C Δ 21, respectively, into microtubules (Fig. 5A). A 22-amino acid truncation of the β III C terminus was incorporated into microtubules in only $22.6 \pm 7.5\%$ of the transfected cells (Fig. 5A). The transition from microtubule-forming to non-microtu-

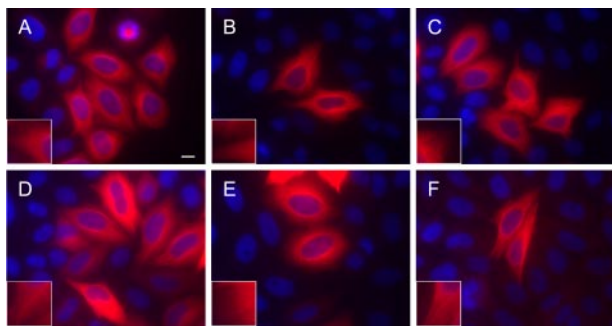


FIGURE 4. Chimeras of β I and β III tubulin do not require the opposing, full-length, C-terminal tail for microtubule incorporation. Chimeras of human β I and β III tubulin were constructed and transfected to show that beyond Ala⁴²⁸ (N-QQYQDA⁴²⁸), the C-terminal tails are interchangeable and do not abolish microtubule formation. Immunofluorescence of HeLa cells transfected with β III+C β I FL (A), β I+C β III FL (B), β I+C β III Δ 5 (C), β I+C β III Δ 9 (D), and β I+C β III Δ 13 (E) indicates that all chimeric constructs enter microtubules. A chimera, β I+C β III FL (F), was created lacking the V5 tag to show that the tag does not interfere with microtubule formation. The cells in A–E were stained with antibody to the V5 tag; the cells in F were stained with the antibody SDL.3D10 to β III. Twenty-four hours post-transfection, the cells were fixed and labeled with an antibody to V5 tag followed by Cy3-conjugated goat anti-mouse and visualized at 60 \times magnification. *Insets*, visualization of cortical microtubules after 2 \times digital expansion. Microtubules are shown in red, and nuclei are shown in blue. Notice that the full-length, chimeric tail is not required for microtubule formation (C–E). Bar, 10 μ m.

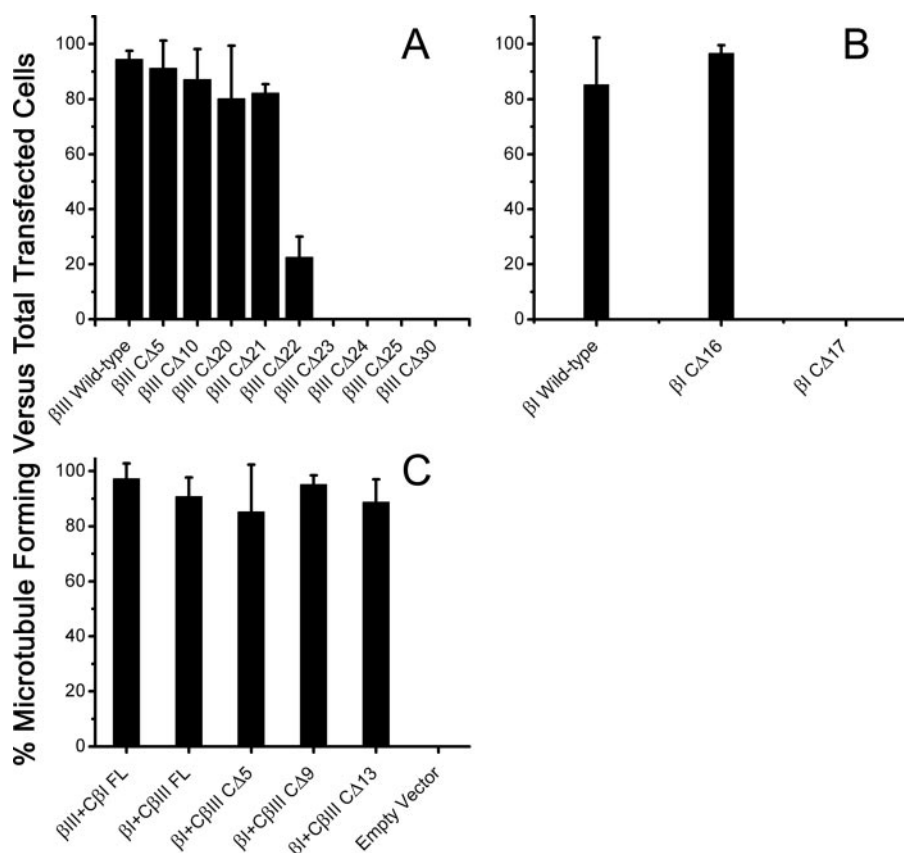


FIGURE 5. Quantitative analysis of HeLa cells transfected with β I, β III, and chimeric β -tubulin constructs were calculated with respect to microtubule-forming versus non-microtubule-forming. Immunofluorescence of transiently transfected HeLa cells were visualized at 60 \times magnification and quantitated based on the ability of each transfected construct to enter microtubules. β III tubulin truncations (A), β I tubulin truncations (B), and chimeras (C) were counted and expressed as percentages of microtubule-forming versus non-microtubule-forming cells. For each construct, four random fields were averaged with standard deviation. Notice that β III Δ 23 and β I Δ 17 (A and B, respectively) do not form any microtubules. In addition, all chimeras, including truncated chimeras β I+C β III Δ 5, β I+C β III Δ 9, and β I+C β III Δ 13, formed microtubules robustly (C). Empty vector did not form microtubules (C).

bule-forming was abrupt and absolute, occurring with the removal of the last 23 amino acids from β III. Although the β I inflection point was the same, ending with the sequence N-QQYQDA⁴²⁸, quantitation between the microtubule forming (β I Δ 16) versus non-microtubule-forming (β I Δ 17) construct was even more abrupt (96.6 ± 2.9 and 0%, respectively) than was the case with β III (Fig. 5B). This demonstrated that the extent of incorporation of different truncated forms varies greatly between β I and β III tubulin. Specifically, the presence of Thr⁴²⁹ strongly promotes microtubule formation in β III but makes absolutely no difference to β I. For both β I and β III, deletion of the critical Ala⁴²⁸ completely abolished microtubules for both isotypes as expected. In addition, all of the chimeras, full-length or otherwise, assembled into microtubules as expected with the following transfects: β III+C β I FL, β I+C β III FL, β I+C β III Δ 5, β I+C β III Δ 9, and β I+C β III Δ 13 having 97.2 ± 5.6 , 90.8 ± 6.9 , 85.2 ± 17.2 , 95.2 ± 3.3 , and $88.8 \pm 8.3\%$ of its cells making microtubules (Fig. 5C).

Deletion of the C-terminal Tail Does Not Affect Dimerization—The results described above indicate that incorporation into microtubules requires that the first 428 amino acids be present. Whether they are also required for incorporation into the tubulin dimer is a separate question. To address this, co-

immunoprecipitation experiments were performed in which the antibody to the V5 tag was used to precipitate the β -tubulin construct to see whether α -tubulin was also precipitated. Expression analysis was performed for wild type, CTT-deleted constructs, and chimeras of β I and β III tubulin (Fig. 6, A–C, top panels). Western analysis with anti-V5 antibody indicated that expression of the transgene was present for all transiently transfected cell lines. Glyceraldehyde-3-phosphate dehydrogenase was utilized as a loading control (Fig. 6, A–C, middle panels). The results of immunoprecipitation clearly showed that truncated tubulins lacking of up to 30 amino acids for β III tubulin (Fig. 6A) and 17 amino acids for β I tubulin (Fig. 6B) were all able to precipitate α -tubulin. The same was true for the chimeric β -tubulins (Fig. 6C). This suggests that truncated forms of these isotypes were able to form $\alpha\beta$ heterodimers.

DISCUSSION

The C-terminal region of tubulin, here defined as the region downstream of the empirically determined Helix H12, is an intriguing and not well understood region of the tubulin molecule. The structure

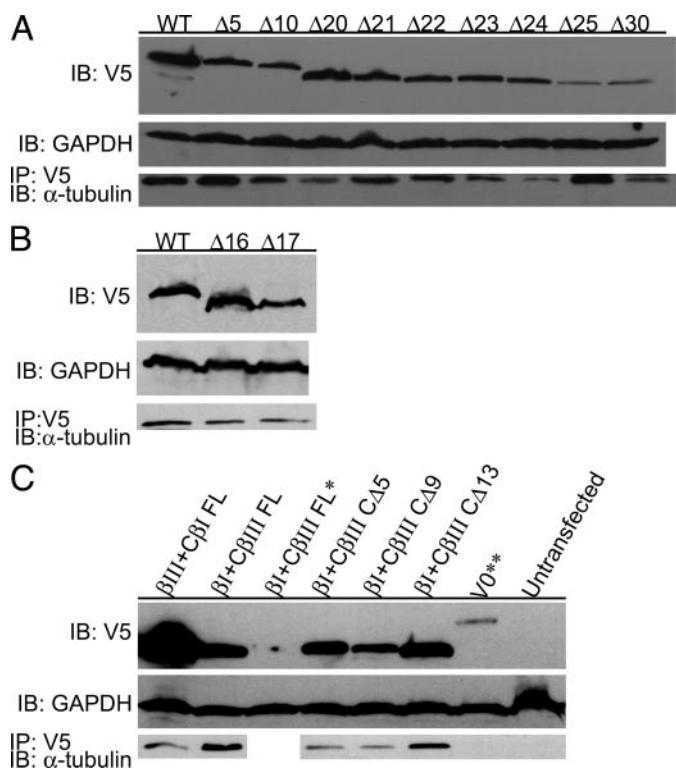


FIGURE 6. Co-immunoprecipitation and Western analysis of β I, β III, and chimeric β -tubulin constructs. HeLa cells were transiently transfected with wild type or the indicated C-terminally truncated β III tubulin (A), β I tubulin (B), or chimeric β -tubulin (C). Expression of β I, β III, and chimeric β -tubulin in HeLa cells were analyzed by Western blotting with antibody to V5 (A–C, top panel) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the loading control (A–C, middle panel). The samples were immunoprecipitated and analyzed by Western blotting with antibody to α -tubulin (A–C, bottom panels). C, *, β I + C β III FL construct that does not contain C-terminal V5-His tag. **, empty vector expressing V5-His-tagged β -galactosidase. Notice that β I C Δ 17, β III C Δ 23, β III C Δ 24, β III C Δ 25, and β III C Δ 30 were all able to precipitate α -tubulin. IB, immunoblot.

of the C-terminal region has not been determined by any kind of crystallographic analysis, although its secondary structure has been modeled (45). This region is known to be a binding site for microtubule-associated proteins and motors (11–22) and to be the site of a variety of important post-translational modifications including phosphorylation, polyglycylation, polyglutamylation, tyrosination/detyrosination, and deglutamylation (23–29, 32, 33, 35–38, 41). Our results raise the possibility that part of the C-terminal region in β -tubulin may have yet another function, namely to create or maintain a conformation in the rest of the tubulin molecule that permits microtubule assembly. It is clear from our results that Ala⁴²⁸ is absolutely required for microtubule formation for both β I and β III. When Ala⁴²⁸ is removed, no microtubules form. The overall structure of the rest of the tubulin is likely to be perturbed as shown by the formation of aggregates that accompanies a lack of microtubule formation. The nature of the C terminus beyond residue 430 appears to have little effect, as indicated by the fact that the chimeric tubulins form microtubules normally.

It is interesting that β I and β III differ in the roles of the residues beyond Ala⁴²⁸. The presence or absence of Thr⁴²⁹ makes no difference at all to assembly of β I. However, the absence of Thr⁴²⁹ strongly inhibits formation of microtubules

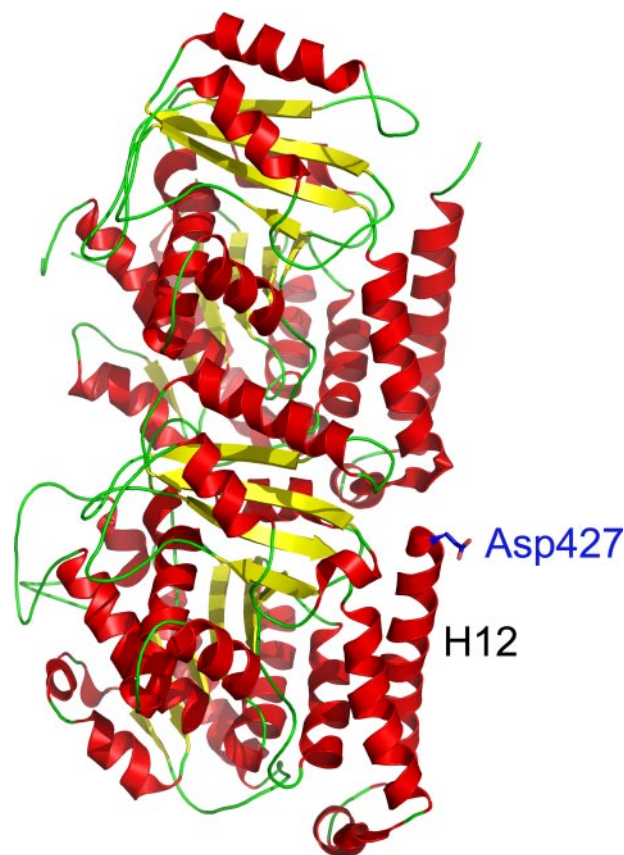


FIGURE 7. Location of Asp⁴²⁷ at the C terminus of β -tubulin. This representation of the α - β -tubulin dimer, taken from the structure by Lowe *et al.* (5) (Protein Data Bank code 1JFF), indicates the location of Asp⁴²⁷ near the end of helix H12. The Asp⁴²⁷ side chain of β -tubulin is shown in blue. This structure was generated using PyMOL (72).

from β III. Even removal of Ala⁴³⁰ appears to slightly inhibit microtubule formation in β III (Fig. 5A).

One could argue that the V5 reporter group, which consists of 45 residues, could create artifactual results in that its bulk could either provide inertial force or steric hindrance that could alter the natural properties of the tubulin molecule. In the former case one could imagine that the bulky V5 group might cause some partial unfolding of the globular domain, just enough to destabilize its conformation and prevent the tubulin molecule from incorporating into the microtubule. In the latter case, one could imagine that, as the V5 group might physically block the tubulin-tubulin interactions that are required for microtubule assembly and that, hence, the tubulin molecule involved would not be able to incorporate into the microtubule. One could further imagine that in a series of truncated mutants, either of these effects would occur at a specific chain length.

There are several arguments that militate against either of these hypotheses and indicate that the results reported here are not likely to be artifacts because of the presence of the V5 group. First, the inertial force hypothesis is weakened by the fact that the projecting C-terminal domain is really quite long, beginning at residue 427 (shown in Fig. 7). It is difficult, although not impossible, to imagine that the putative inertial force exerted by the V5 domain would be such that at a residue exactly 23 or 17, for β III or β I, respectively, positions into the C-terminal domain, but presumably well removed from the globular

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domain, the inertial force would suddenly make a difference and cause partial unfolding of the globular domain. Second, it is interesting that the transition from allowing microtubule assembly to preventing it occurs at the precise same residue in βI as in βIII . The inertial force hypothesis presupposes that the globular domain exerts some force on the C-terminal domain that is overcome when the V5 group approaches just close enough to the globular domain. It is unlikely that the globular domains of βI and βIII would exert the exact same force on the C-terminal domain such that removal of the exact same residue on βI and βIII would cause destabilization. Although the stability of βI has never been measured directly, the stabilities of βII , βIII , and βIV have been measured. βIII is far more stable than βII , and βII is much more stable than βIV (45, 46). βI is very similar in sequence to βIV (Table 1), in both its globular domain and C-terminal domain and is therefore likely to be much less stable than βIII . In short, if the inertial force hypothesis were to be correct, the transition in βI from permitting to preventing microtubule formation would probably not occur at the same residue as it does in βIII .

Although this argument may rule out the inertial hypothesis as a source of artifact, they do not rule out the steric hindrance hypothesis, namely that V5 could be blocking assembly once the distance between the V5 domain and the globular domain of tubulin becomes sufficiently short and that the critical truncation is the one that removes Ala⁴²⁸. If that were the case, then there is no reason to expect βI and βIII to give different effects. The effect should simply be a matter of length. However, our finding that Thr⁴²⁹ strongly promotes the ability of βIII to form microtubules but makes no difference for βI argues against the steric hindrance hypothesis. By either argument, the fact that the βI chimeric mutant containing the βIII C terminus ($\beta I + C\beta III$ FL) but lacking the V5 tag, assembles perfectly well into microtubules (Fig. 4F) suggests that the 45-residue V5 tag is not influencing the results and corroborates our finding that the nature of the C-terminal region beyond Thr⁴²⁹ has little or no effect on the likelihood of microtubule incorporation.

There are only two mechanisms by which the C-terminal domain can regulate the ability of tubulin to form microtubules. Either the C-terminal domain spends at least a fraction of the time lying on the surface of the globular domain and maintaining its conformation so that it can enter the microtubule or the C-terminal domain somehow reaches over to another tubulin molecule, bonding with it so as to stabilize the microtubule. If the latter mechanism is correct, then the isotopic nature of the C-terminal domain should make no difference, because it is known that different isoforms of tubulin can co-polymerize (47). Our data indicate that there is no specific relationship between the isotopic nature of the C terminus and that of the globular domain, because βIII with the βI C terminus and βI with the βIII C terminus polymerizes into the microtubule. Hence, our data support the second mechanism.

Although the larger C-terminal truncations beyond Ala⁴²⁸ did not assemble into microtubules, our data suggest that the C-terminal domain and even part of Helix H12 play no part in maintaining $\alpha\beta$ dimer formation. This is not surprising, because the crystal structure (5) indicates that the dimer-forming region is not near the CTT. The C-terminal domain of tubu-

lin has already been postulated to serve as a signal, as a site for binding of MAPs and motors, and as a site for post-translational modifications, all of these with great functional significance. Eliminating this region is likely to cause severe alterations in dynamic behavior. Because immunoprecipitation suggests that α - and β -tubulin are associated, the fluorescence data from microtubule inhibiting truncations are likely a display of the free dimer pool. Perhaps these deletions, lacking the C terminus, have caused a shift to a higher critical concentration for microtubule assembly.

The differences between βI and βIII are highly conserved in evolution, implying some degree of functional significance to those differences (1). It is striking that βIII lacks Cys²³⁹ and contains Cys¹²⁴. Cys²³⁹ is present in all of the vertebrate β isoforms (except for βV and βVI). It is also present in all other animal β isoforms and in most plant and protist (but not fungal) β isoforms. A few plants and protists have Cys²³⁸ instead of Cys²³⁹, some have both, but, outside of vertebrate βIII , βV , and βVI and fungal β -tubulins, there is always a cysteine in that area (1). Cys¹²⁴ is even more striking. It is present in βIII , βV , and βVI , but not in any other β -tubulin (except for octopus (48)). It is likely that βIII may have a unique function that involves these particular residues. It is known that βIII has a conformation different from that of either βII or βIV , the latter, as already argued, being very likely to have a conformation very similar to that of βI (46, 49). It is likely, therefore, that the conformations of the globular domains of the intact βI and βIII molecules are different and that, in the case, of βIII , this may permit βIII to carry out its function. It should be noted that the presence of $\alpha\beta III$ dimer in the outer mitochondrial membranes of certain tumor cells raises the possibility that βIII may perform functions when it is not part of the microtubule (50). One could even speculate that a potentially different conformation of βIII may require slightly more of the C-terminal region (e.g. Thr⁴²⁹) to, in a sense, "corral" the conformation of βIII into one consistent with microtubule assembly.

A survey of β -tubulin sequences indicates that Ala⁴²⁸ is universally conserved. However, the same is not true of Thr⁴²⁹. Thr⁴²⁹ occurs in all of the human β -tubulin isoforms except for βVI . It also occurs in a variety of protists (e.g. *Moneuplotes* (51) and *Paramecium* (52)), fungi (e.g. *Trichoderma* (53)), plants (e.g. lupine (54), *Eleusine* (55), carrot (56), and tobacco (57)), and animals (e.g. the nematode *Cooperia* (58), the sea urchin *Strongylocentrotus* (59), the squid *Loligo* (60), the Antarctic fish *Notothenia* (61), *Xenopus* (62), the chicken (63), and the mouse (64)). However, Thr⁴²⁹ is replaced by valine in cotton (65), by serine in the moss *Physcomitrella* (66), and by glycine in the platyhelminth *Echinococcus* (67), the parabasalid *Trichonympha* (68), and *Giardia* (69). Interestingly, in the human βVI isoform, Thr⁴²⁹ is replaced by a lysine (National Center for Biotechnology accession number CAC09371). The other replacements of Thr⁴²⁹ involve small amino acids. Lysine is considerably larger. Conceivably, Lys⁴²⁹ may play a role in the formation of the unusual microtubules of blood platelets, which have a high propensity to form curved structures that constitute the marginal bands of these cells (70, 71). In short, our results are consistent with the phylogenetic distributions of Ala⁴²⁸ and Thr⁴²⁹; Ala⁴²⁸ is absolutely required for microtubule assembly

because it is absolutely conserved in evolution, and Thr⁴²⁹ strongly promotes microtubule assembly in at least one but not all types of tubulin because it is strongly but not absolutely conserved in evolution.

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