# A Monoclonal Antibody to α-Tubulin: Purification of Functionally Active α-Tubulin Isoforms†

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ABSTRACT: Both α- and β-tubulin exist as numerous isotypic forms that originate from different primary sequences as well as a variety of posttranslational modifications. Recent studies show that tubulin dimers differing in the β-subunit differ significantly in their subcellular distribution as well as in their functional properties such as assembly, dynamics, conformation, and interaction with antimitotic drugs; however, very little is known about the functional significance of the different α-tubulin isoforms and their posttranslational modifications. In an effort to get a better understanding about the α-tubulin isoforms, a monoclonal antibody, AYN.6D10, was prepared against the mammalian α-tubulin C-terminal sequence Glu-Glu-Gly-Glu-Glu-Tyr. Using an immunoaffinity column, bovine brain tubulin was fractionated into three functionally active αβ heterodimers which were identified by immunoblotting with α-tubulin-specific antibodies and sequence analysis. Assembly studies in the presence of glycerol and $Mg^{2+}$ show that one of the fractions, that contains mainly the tyrosinated form of α1/2, assembled poorly, while the nontyrosinated form assembled normally. The results indicate that tubulin dimers differing in their α-tubulin may differ in their functional properties. Future studies with the isoforms may yield valuable information regarding the role of α-tubulin and its posttranslational modifications in regulating microtubule assembly and function in vivo.

Microtubules, the ubiquitous eukaryotic organelles, mediate various cellular functions such as cell division, motility and transport, maintenance of cell shape, and signal transduction (*1*, *2*). Tubulin, the major subunit of microtubules, is a heterodimeric protein consisting of two related peptides known as α- and β-tubulin (*1*, *2*). Both α- and β-tubulin exist as multiple isoforms which differ in their primary sequences as well as in the posttranslational modifications (*3*−*12*). In mammalian species, about six α-tubulin classes are encoded by the genes Mα1, Mα2, Mα3/7, Mα4, Mα6, and αTT1 (*3*−*12*) (the prefix "M" indicates that these genes were originally identified in the mouse). On the other hand, there are seven β-tubulin classes, designated as $\beta_{I}$, $\beta_{II}$, $\beta_{III}$, $\beta_{IVa}$, $\beta_{IVb}$, $\beta_{V}$, and $\beta_{VI}$, which have unique C-terminal sequences (*5*, *7*) [for reviews on tubulin isoforms, see (3), (7), and (9)]. The polypeptides α1 and α2 differ only at amino acid 232 (Gly/Ser) and are expressed mainly in the brain and also to some extent in other tissues; α3/7 is a form that is encoded by two different genes, α3 and α7, which are expressed only in the testes; α4 is unique in the sense that it lacks the coded C-terminal tyrosine residue, and is expressed constitutively in many tissues but mainly in the brain, muscle, and heart; αTT1 is found only in the testes (*6*); α6 is expressed in the liver and stomach. Since α1 and α2 differ in just one amino acid, they will be grouped here as α1/2. Of these five different α-tubulin classes, α1/2 and α4 are the α-tubulins found in the brain (*5*). It is known that α1/2 and α4 both occur in birds (*3*, *7*, *9*), suggesting that the differences among them have been conserved at least since the divergence of the mammalian and avian lines 310 million years ago. The strong conservation of these differences suggests that they are functionally significant. Although the α-tubulin classes do not have unique C-terminal sequences as do the β-tubulin classes, significant sequence differences are found among different α-tubulins. The C-terminal sequences for different α-tubulin classes are depicted in Figure 1.

Tubulin undergoes posttranslational modifications that include (a) tyrosination and detyrosination of α-tubulin (*13*, *14*); (b) acetylation of α-tubulin at $Lys^{40}$ (*15*, *16*); (c) loss of both the C-terminal tyrosine and the penultimate glutamic acid residue from α-tubulin, resulting in the formation of Δ2 tubulin[1] (*17*); (d) polyglutamylation of both α- and β-tubulin, in which glutamic acid residues are added laterally through a γ-carboxyl of a glutamic acid at the C-terminal region through an isopeptide linkage (*18*−*20*); (e) polyglycylation of both α- and β-tubulin (*21*); and (f) phosphorylation of the $\beta_{III}$ isoform (*22*−*24*).

To understand the roles each tubulin isoform can play in vivo, it would be necessary to purify an isoform in the functionally active form. Thus, we have generated monoclonal antibodies specific for the $\beta_{II}$, $\beta_{III}$, and $\beta_{IV}$ isoforms using the C-terminal peptides corresponding to each isoform (*25*−*27*). Using these antibodies on immunoaffinity columns, the β-tubulin isoforms $\alpha\beta_{II}$, $\alpha\beta_{III}$, and $\alpha\beta_{IV}$ have been

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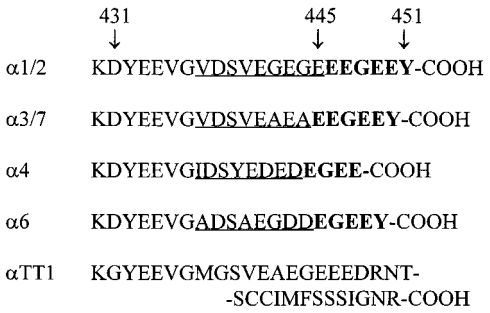


FIGURE 1: C-terminal sequences for the mammalian α-tubulin classes. The sequences from residue 430 through the C-termini are shown. The glutamic acid residue at 445 (indicated by an arrow) is the residue that is the site of posttranslational polyglutamylation. The sequence differences among different classes are underlined. The antigenic sequence used for making the antibody AYN.6D10 is in boldface letters.

purified in functionally active forms (*27*). The studies with purified *β*-tubulin isoforms reveal that they differ significantly in drug binding (*28*−*30*), drug-induced conformational changes (*31*), assembly (*27*), and in the dynamics of the microtubules they form (*32*). Although ample evidence has accumulated to demonstrate that the *β*-tubulin isoforms indeed differ in their functional properties, no such information is available on α-tubulin. One major reason may be that it has not been possible to make a monoclonal antibody specific for any α-tubulin class. Thus, this project was initiated to generate monoclonal antibodies specific for different α-tubulin classes. I have generated a monoclonal antibody by using a synthetic peptide corresponding to the C-terminal hexapeptide (EEGEEY) of most α-tubulins (Figure 1). By using the immunoaffinity column, bovine brain tubulin was fractionated into four fractions according to the nature of their α-subunit. In vitro assembly studies were carried out to study the functional differences among different α-tubulin fractions.

## EXPERIMENTAL PROCEDURES

*Materials.* The C-terminal peptide for the α-tubulin isoform $NH_2$-Cys-Glu-Glu-Gly-Glu-Glu-Tyr-COOH was custom-synthesized by Genosys Biotechnologies, Inc., Woodlands, TX. The cysteine residue at the $NH_2$-terminal is required for coupling the peptide with the carrier protein. Freund's adjuvants, maleimide-activated KLH, or BSA was obtained from Pierce. Poly(ethylene glycol) (PEG 1500) was obtained from Boehringer-Mannheim. Fetal bovine serum was from Atlanta Biologicals (Norcross, GA). Carboxypeptidase A and the mouse monoclonal antibody to tyrosinated tubulin (clone TUB.1A2) were obtained from Sigma. The polyclonal antibody to Glu tubulin was a kind gift from Drs. J. C. Bulinski and G. G. Gunderson, Columbia University, New York, NY. HRP-labeled antibodies were obtained from Pierce. The chemiluminescent substrate (Enhanced NuGlow) for immunoblot development was obtained from Alpha Diagnostic Intl. Inc., San Antonio, TX.

*Coupling of the Antigen with the Carrier Protein*. The peptide was coupled to KLH or BSA according to the instructions provided by the manufacturer. Three milligrams of the peptide dissolved in 40 *μ*L of dimethyl sulfoxide was incubated with 300 *μ*L of maleimide-activated BSA or KLH (10 mg/mL) at the room temperature for 2 h. The mixture was diluted to 3 mL and then dialyzed for 14 h against sterile PBS using sterile dialysis tubing. The peptide was stored in aliquots at −20 °C.

*Immunization of Mice and Preparation of the Monoclonal Antibody*. Two female balb/c mice (4 weeks old) were injected initially with the KLH-peptide (approximately 100 *μ*g) in Freund's complete adjuvant. After 2 weeks, the mice received a second injection with Freund's incomplete adjuvant. After 10 days, the mice were bled through the tail, and the sera were checked for antibody titer in an ELISA using bovine brain PC−tubulin. None of the mice exhibited positive. After 10 days, the mice were injected with peptide−BSA in the incomplete adjuvant. After 10 days, the mice were bled, and the antibody titer was checked. Both mice exhibited significant titer even at a serum dilution of 1:5000. After 10 days, one of the mice was injected intraperitoneally with the peptide−BSA without adjuvant. After 3 days, the mouse was sacrificed, and the spleen was removed. The spleen cells were fused with SP2/0 myeloma cells in the presence of 50% PEG 1500. After the fusion, the cells were diluted in 200 mL of HAT selection medium containing 20% fetal bovine serum, and were subsequently plated in 20, 96-well culture plates and kept in a humidified $CO_2$ incubator for 1 week. The colonies started appearing after 1 week. The colonies were checked for antibody titer in ELISA assay with PC−tubulin. Out of 1920 fusion wells, only 278 had colonies in them while only 7 showed positive against brain tubulin. These seven clones were single-cell-cloned on gamma-ray-irradiated rat thymocytes. Out of these seven antibodies, four were of IgM type, one was $IgG_1$, and two were $IgG_{2b}$. One clone (AYN.6D10) was grown up in roller bottles, and the monoclonal antibody was purified on a protein A−agarose column. Isotyping shows that the antibody is an $IgG_{2b}$ having both light chains of the *κ*-type.

*Preparation of the E. coli Fusion Proteins Containing the C-Terminal Sequence of Each α-Tubulin Class*. The fusion proteins (kind gifts from Drs. Nicholas J. Cowan and Sally Lewis, New York University Medical Center, New York, NY) were made by fusing the *E. coli* tryptophan synthetase gene (trp E) with the cDNA sequence corresponding to the C-terminal portion of each α-tubulin (*33*). For the α-tubulin classes α1/2, α3/7, α3/7 −Y, and α6, the C-terminal portion included residue 254 to the C-terminal end; for α4 and α4 +Y, the portion included residue 168 to the C-terminal end. Thus, the fusion proteins for α4 and α4 +Y are larger in size as compared to the others. The C-terminal tyrosine residue was added to α4 by site-directed mutagenesis of a termination codon to one that codes for a tyrosine residue.

[1] Abbreviations: α3/7 −Y, α3/7 tubulin lacking the tyrosine residue at the C-terminus; α4 +Y, α4 tubulin with an additional tyrosine residue at the C-terminus; BSA, bovine serum albumin; buffer A, 0.1 M MES-Na, pH 6.4, 1 mM EGTA, 0.1 mM EDTA, 0.5 mM $MgCl_2$, and 1 mM GTP; EDTA, ethylenediaminetetraacetate; Δ2 tubulin, α-tubulin lacking the C-terminal tyrosine and glutamic acid residues; EGTA, ethylene glycol bis(*β*-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetate; ELISA, enzyme-linked immunosorbent assay; Glu tubulin, α-tubulin lacking the C-terminal tyrosine residue; HPLC, high-performance liquid chromatography; HRP, horseradish peroxidase; KLH, keyhole limpet hemocyanin; MES, 2-(*N*-morpholino)ethanesulfonic acid; PC-tubulin, tubulin purified by phosphocellulose chromatography; PBSTT, phosphate-buffered saline containing 0.02% thimerosal and 0.1% Tween-20; SDS−PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; TFA, trifluoroacetic acid; Tyr tubulin, α-tubulin having a tyrosine residue at the C-terminus.

Similarly, the C-terminal tyrosine residue was removed from the α3/7 sequence to form the α3/7 −Y sequence.

*Preparation of Tubulin.* Microtubules were purified from bovine brain cortex by a cycle of assembly and disassembly, and tubulin was purified from microtubules by phosphocellulose chromatography as described elsewhere (*34*). All purifications were carried out in buffer A that contains 0.1 M MES-Na, pH 6.4, 1 mM EGTA, 0.1 mM EDTA, 0.5 mM $MgCl_2$, and 1 mM GTP.

*Preparation of α-Tubulin Fractions for Functional Studies.* Tubulin fractions obtained from the immunoaffinity column were identified by a Bradford protein assay, pooled separately, and concentrated on Amicon ultrafiltration unit. The fractions were dialyzed against buffer A to remove NaCl, were concentrated by overnight (14 h) dialysis in buffer A containing 8 M glycerol, and were stored frozen in aliquots at −80 °C in buffer A (0.1 M MES-Na, pH 6.4, 1 mM EGTA, 0.1 mM EDTA, 0.5 mM $MgCl_2$, and 1 mM GTP) containing 8 M glycerol. Glycerol was removed before each experiment by repeated dilution and centrifugation using Centricon 30 membrane filtration units.

*Preparation of the Immunoaffinity Column*. The antibody AYN.6D10 was coupled to CNBr-activated Sepharose according to the instructions provided by Pharmacia for CNBr-activated Sepharose 4B as described in Banerjee et al. (*25*). About 63 mg of antibody was incubated with 10 g of CNBr−Sepharose 4B at 4 °C for 12−16 h with gentle shaking. The unoccupied sites were blocked by overnight incubation with 1.0 M ethanolamine hydrochloride (pH 9.0). The resin was subsequently subjected to three cycles of alternating pH washes (sodium acetate buffer, pH 4.0, and Tris-HCl buffer, pH 8.0). The resin was finally resuspended in 0.1 M MES buffer (pH 6.4) and was stored with 0.02% sodium azide.

*Gel Electrophoresis and Immunoblotting*. Gel electrophoresis was carried out in polyacrylamide gels (10 cm long) in the presence of sodium dodecyl sulfate according to Laemmli (*35*). Tubulin samples were reduced and carboxymethylated (*36*) prior to the run. Protein bands from the gels were transferred onto a nitrocellulose membrane using a BIO-RAD Trans-Blot cell as described elsewhere (*25*). For immunoblotting, the nitrocellulose blots were blocked in 5% Carnation nonfat milk and 0.1% BSA in PBSTT buffer for 2 h, incubated with the primary antibody for 1 h, and washed 3 times with PBSTT with mild shaking. The blots were incubated with HRP−antibody for 1 h followed by 3 washes with PBSTT. The blots were developed using chemiluminescent substrate and were exposed immediately for 5 min on KODAK X-Omat AR film.

*Preparation of Mouse Polyclonal Antiserum to α4 Tubulin.* Mouse polyclonal antiserum to α4 tubulin was prepared by repeatedly immunizing 3−4 week old female balb/c mice with a synthetic peptide, Ile-Asp-Ser-Tyr-Glu-Asp-Glu, corresponding to the α4 tubulin sequence (amino acid residues 437−443). Each mouse was injected subcutaneously with about 100 μg of the BSA-conjugated peptide in PBS buffer along with the Freund's adjuvants. After four immunizations, the mice were bled through their tails and the sera were collected.

*Preparation of α-Tubulin for Sequencing Studies.* The details of the steps leading to sequence analysis are outlined in Figure 7. The protein samples (without carboxymethylation) were boiled for 5 min with an equal volume of 2× Laemmli sample buffer and were run on preparative SDS gels (using SDS from Sigma)[2] (6%, 5 mm thick) to separate α- and β-tubulin. About 500 μg of tubulin samples was run at a time. After the electrophoresis, the tubulin bands were visualized either by staining and destaining or by immersing the gel in ice-cold 4 M sodium acetate. The protein bands were cut out and were taken in glass tubes (10 × 1 cm), and the protein was electro-eluted according to Rushbrook (*37*) using a vertical tube gel apparatus. Elution was carried out at room temperature for 14 h at a constant current of 10 mA per tube using Laemmli running buffer, and the eluted protein was collected in dialysis tubing. The protein was precipitated with 10 volumes of superchilled acidified acetone (acetone:1 N HCl, 40:1, v/v) to remove the SDS, dissolved in 0.1 M Tris HCl (pH 9.0). This cycle was repeated twice, and finally the protein was dialyzed in the above buffer for sequencing.

*Sequence Analysis.* Protein samples in 0.1 M Tris-HCl (pH 9.2) were digested with endoproteinase Lys-C (17 μg/mL) at room temperature for 14 h. The enzyme cleaves at the C-terminal peptide bond of each lysine residue. For α-tubulin, the cleavage at the lysine residue ($Lys^{430}$) is readily achieved, leaving 18−21 amino acid long C-terminal peptides (*38*). Fifteen micrograms of the tubulin digest was subjected to reversed-phase HPLC on an Applied Biosystems ABI 130 HPLC using a Phenomenex Atlantis C18 column (50 × 2 mm). Solvent A was 0.1% TFA in water; solvent B was 0.085% TFA in 70% acetonitrile in water. The chromatography was performed using a gradient of 0−60% solvent B in 60 min at a flow rate of 0.5 mL/min. The peaks containing the C-terminal fragments were identified by sequence analysis. Sequencing was performed on an Applied Biosystems 477 A protein sequencer by the automated Edman degradation method using aryl-covalent membrane (Millipore). PTH-amino acids were separated using ABI 120 HPLC.

*Other Methods.* Microtubule assembly and enzyme-linked immunoassays (ELISA) were performed as described in Banerjee et al. (*25*). Protein measurements were done according to Lowry et al. (*39*). For column-eluted fractions, protein was measured according to the dye-binding method of Bradford (*40*).

## RESULTS

*Characterization of the Monoclonal Antibody AYN.6D10 by Immunoblot Analysis.* The antibody was tested against bacterial fusion proteins corresponding to the C-terminal sequences of different α-tubulin classes by SDS−PAGE and immunoblotting. As shown in Figure 2, the antibody recognized α1/2, α3/7, α6, and α4 +Y. The α-tubulin classes that were not recognized by the antibody are Mα4 and Mα3/7 −Y, both of which lack the C-terminal tyrosine. In an attempt to study whether the C-terminal tyrosine is the epitope for the antibody, a detyrosination experiment was performed. For this, the fusion proteins were subjected to SDS−PAGE as before, and the gel was transferred onto a nitrocellulose membrane. The blot was subsequently treated with carboxypeptidase A (4 μg/mL, 2 h) to remove the C-terminal tyrosine from the fusion proteins and was

[2] For unknown reason, the SDS purchased from Sigma Chemical Co. gives the best separation of α- and β-tubulin bands for samples which were not reduced and carboxymethylated.

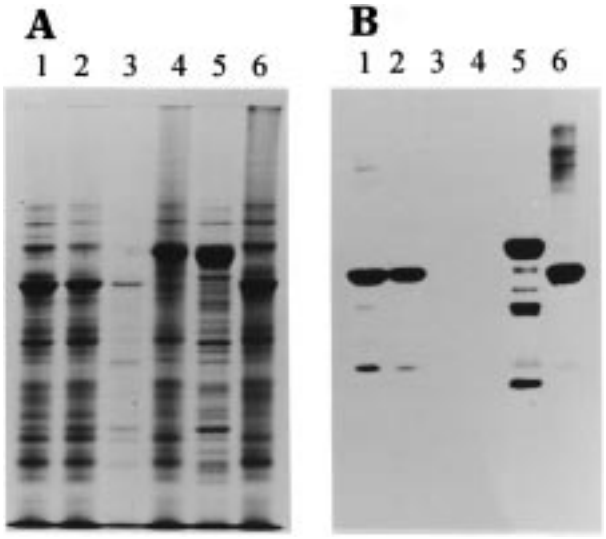


FIGURE 2: Specificity of monoclonal antibody AYN.6D10. *E. coli* extracts containing trp E fusion proteins corresponding to the C-terminal portion of Mα1/2 (lane 1), Mα3/7 (lane 2), Mα3/7 −Y (lane 3), Mα4 (lane 4), Mα4 +Y (lane 5), and Mα6 (lane 6) were subjected to SDS−PAGE (8%). An identical gel was transferred onto a nitrocellulose membrane and was processed for immunoblotting using AYN.6D10 as the primary antibody (1:10 000 dilution), HRP-labeled secondary antibody, and an enhanced chemiluminescent substrate. Panel A: Coomassie blue stained gel. Panel B: blot. Notice that the antibody did not react with the fusion proteins that lacked a C-terminal Tyr residue. Minor bands in the blot are due to the degradation of fusion proteins in the bacterial extract, which is almost unavoidable.

subsequently tested by immunoblotting. The results showed that none of the lanes were recognized by the antibody (data not shown). When the same blot was treated with the anti-Glu tubulin antibody, all the lanes were lighted up. Thus, the results of the immunoblot analysis showed that the monoclonal antibody AYN.6D10 recognizes all the α-tubulin classes with a C-terminal tyrosine residue and that the antibody recognition is abolished upon removal of the C-terminal tyrosine by digestion with carboxypeptidase A.

*Chromatography of Bovine Brain Tubulin on the Antibody−Sepharose Column.* In an effort to test the tubulin-binding property of the immunoaffinity column, PC−tubulin was fractionated on the column as outlined in Figure 3. After the elution of the unbound fraction (fraction A), the column was washed well with the buffer. The column was then eluted with a linear salt gradient of 0−1 M NaCl. As shown in Figure 4, the bound protein was eluted as two well-separated peaks, fractions B (peak I) and C (peak II). After this gradient elution, the column was finally eluted with 3 M KI to get the remaining bound protein (fraction D). The relative yields for the fractions were calculated from the area under the peaks from Figure 4. Thus, the relative amounts are found to be 50.2%, 29.4%, 14.1%, and 6.3% for fractions A, B, C, and D, respectively. To test the functional activity, the tubulin fractions (except the KI-eluted fraction D) were dialyzed to remove the salt, were concentrated by overnight dialysis in buffer A containing 8 M glycerol, and were stored in aliquots at −80 °C.

*Determination of α4 Content in the Isoform Fractions.* Since bovine brain α-tubulin contains only α1/2 and α4 isoforms, it was necessary to see whether different α-tubulin isoform fractions differ in the composition of α1/2 and α4.

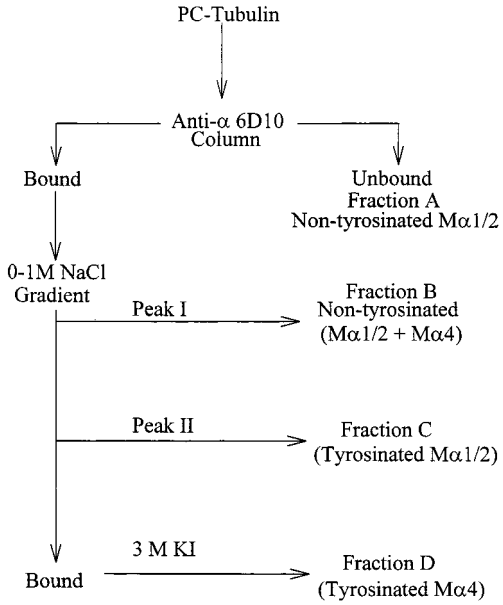


FIGURE 3: Schematic outline for the purification of the α-tubulin isoforms.

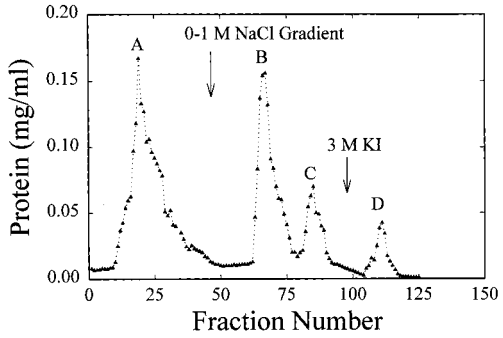


FIGURE 4: Fractionation of bovine brain tubulin on an immunoaffinity column containing the monoclonal antibody AYN.6D10. Bovine brain PC−tubulin (7 mg) was passed through the antibody−Sepharose column (15 mL, containing 30 mg of antibody) equilibrated in buffer A, and the unbound fraction was collected (each fraction was 1.5 mL). The column was subsequently washed with 50 mL of the same buffer, and the bound fraction was first eluted with a linear salt gradient of 0−1 M NaCl. Protein was determined in all the fractions by a Bradford protein assay. Notice that the bound fraction eluted as two peaks (B and C). The two peaks were pooled separately. After the elution of B and C, the remaining bound fraction was eluted with 3 M KI in buffer A.

A polyclonal antiserum against α4 was used to test the presence of α4 in those fractions. The presence of α4 was checked by SDS−PAGE and subsequent immunoblotting analysis with mouse polyclonal anti-α4 antiserum. The result (Figure 5) shows that the NaCl-eluted fraction B and the KI-eluted fraction D light up with the anti-α4 antiserum, while the unbound fraction A and the NaCl-eluted fraction C do not. These data clearly indicate that fractions A and C are completely depleted of α4 and thus contain mainly α1/2.

*Determination of the Posttranslational Modifications in α-Tubulin in the Isoforms.* Since the antibody AYN.6D10 was found to react only with the tyrosinated forms of the α-tubulin classes, it was interesting to see whether the four fractions obtained by immunoaffinity column chromatography differ in their tyrosination states. To determine the tyrosination state of tubulin, immunoblot analysis was

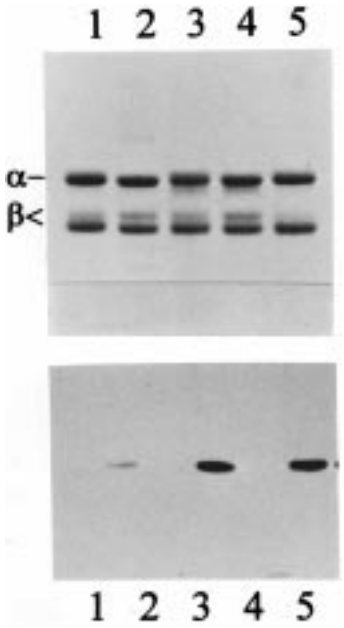


FIGURE 5: Immunoblot analysis of the tubulin isoforms with anti-α4 antiserum. PC−tubulin was passed through the immunoaffinity column as described for Figure 3. After collection of the unbound fraction and subsequent NaCl elution, the remaining bound fraction was eluted with 3 M KI. All the fractions were subsequently reduced and carboxymethylated. The samples were subsequently analyzed by SDS−PAGE (6%) and immunoblotting with the anti-Mα4 antiserum using an enhanced chemiluminescent substrate. Upper panel, Coomassie-stained gel; lower panel, immunoblot. Samples in each lane are as follows: lane 1, PC−tubulin; lane 2, unbound (fraction A); lane 3, fraction B; lane 4, fraction C; and lane 5, fraction D. 2.5 $\mu$g of each sample was loaded in each lane. Note that the anti-α4 antibody recognizes lanes 1, 3, and 5 only, which indicates that Mα4 tubulin is absent in fractions A and C.

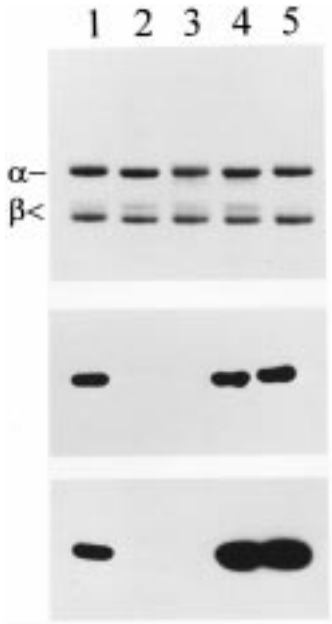


FIGURE 6: SDS−PAGE and immunoblot analysis of the tubulin fractions obtained by immunoaffinity chromatography on the antibody column. Reduced and carboxymethylated tubulin samples were analyzed by SDS−PAGE (6%) and immunoblotting with antibodies to tyrosinated and nontyrosinated tubulin. Top panel, Coomassie blue stained gel; middle and bottom panels, immunoblots developed with an enhanced chemiluminescent substrate. The primary antibodies are middle panel, AYN.6D10; bottom panel, TUB.1A2 (antityrosinated tubulin). The samples in each lane are as follows: lane 1, PC−tubulin; lane 2, unbound fraction A; lane 3, NaCl-eluted fraction B; lane 4, NaCl-eluted fraction C; lane 5, KI-eluted fraction D. 2.5 $\mu$g of each fraction was loaded in each lane.

performed with the monoclonal antibody AYN.6D10 and also with the antibody to tyrosinated tubulin. The results (Figure 6) show that the antibody AYN.6D10 recognized the α-tubulin bands in unfractionated tubulin, and in fractions C and D. No staining was visible for the α-tubulin bands in fractions A and B. None of the $\beta$-tubulin bands were recognized by the antibody. These results indicate that the α-tubulin present in the unbound fraction A and in the NaCl-eluted fraction B is not tyrosinated. Similar results were also obtained when the immunoblotting was done with the monoclonal antibody TUB.1A2 (Sigma) which is specific for tyrosinated tubulin (Figure 6). It should be mentioned here that, although the antibody is specific for tyrosinated tubulin, fraction B that contains the detyrosinated tubulin can still bind to the immunoaffinity column. This is probably due to the nonspecific interaction caused by a very high concentration of antibody in the column (above 3 mg/mL). This will be discussed later under Discussion.

*Sequencing Studies.* To identify the α-tubulin classes in the above fractions, sequence analysis was performed. The details of the procedures leading to sequence analysis are outlined in Figure 7. The α-tubulin bands were cut out from SDS gels and were isolated by electro-elution. The α-tubulin isolated from the above fractions was digested with endoproteinase Lys-C (which cleaves at the C-terminal of each lysine residue), and the fragments were separated by reversed-phase HPLC on a C-18 column. All the fractions were partially sequenced (up to five residues from the $NH_2$-terminal) to identify the C-terminal fragment, which was sequenced

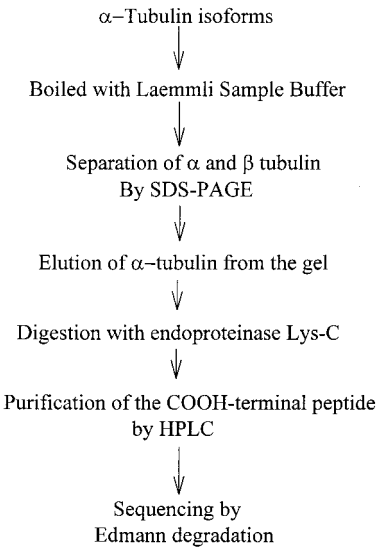


FIGURE 7: Schematic outline for the analysis of different tubulin fractions.

completely. As shown in Figure 8, the C-terminal peptides were eluted at 30−35 min. The unbound fraction (fraction A) showed the two peaks 'a' and 'b' which eluted at around 30−32 min (Figure 8, panel A). Edman degradation of peak 'a' gave the sequence DYEEVGVDSVEGEGEEEGEE, which matches exactly with the C-terminal sequence of α1/2, while peak 'b' gave the sequence DVNAAIATIK ($\alpha^{327-336}$), which is also specific for α1/2. It should be mentioned here

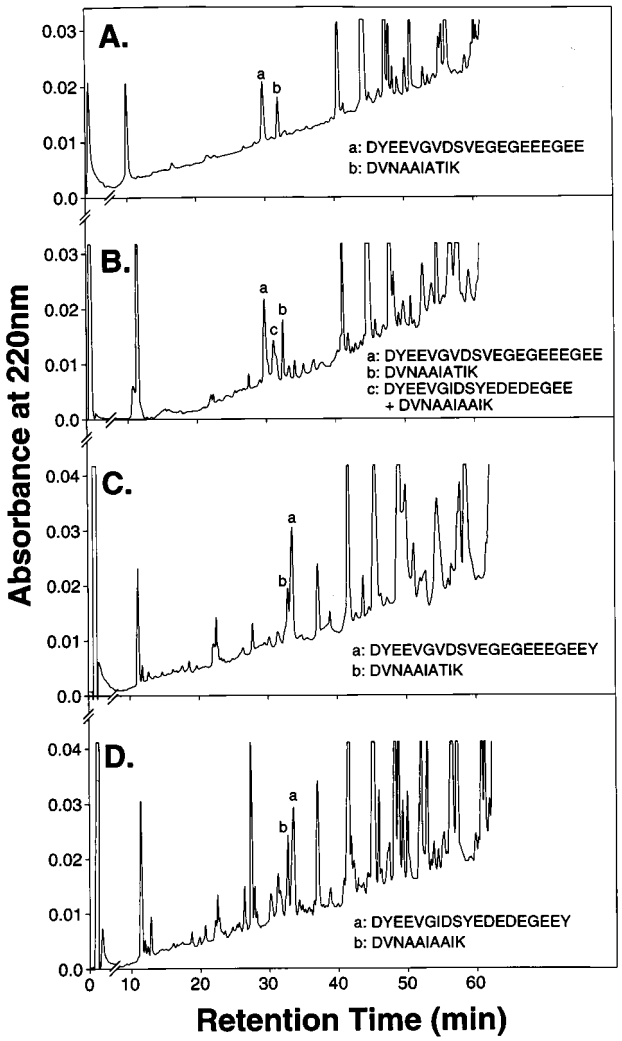


FIGURE 8: HPLC separation of α-tubulin peptides obtained by digesting tubulin fractions with endoproteinase Lys-C. Four tubulin fractions, A, B, C, and D, obtained by fractionating bovine brain tubulin on an AYN.6D10 immunoaffinity column, were subjected to SDS−PAGE on preparative gels, and the α-tubulin bands were cut out and electro-eluted as described under Experimental Procedures. Isolated α-tubulin samples (10 μg) were digested with endoproteinase Lys-C (17 μg/mL) in 0.1 M Tris-HCl (pH 9.2) for 14 h. The tubulin digests were subjected to HPLC using a C-18 column. The C-terminal peptides were identified by sequence analysis using the automated Edman degradation method. Panel A, unbound fraction (fraction A); panel B, NaCl-eluted peak I (fraction B); panel C, NaCl-eluted peak II (fraction C); panel D, KI-eluted fraction (fraction D). The sequences are shown for peptides that are labeled with the letters 'a', 'b', and 'c' in each panel.

that the signals for the amino acid residues $Glu^{445}$, $Glu^{449}$, and $Glu^{449}$ (underlined residues in the above sequence) were significantly low. This will be discussed later. Thus, the unbound fraction seems to be a mixture of the nontyrosinated forms (that may also include the Δ2 form) of α1/2 (the term "non-tyrosinated" is designated for its reactivity with anti-tyrosinated tubulin antibody). All the other peaks were also partially sequenced, and no other peak was found to contain the sequence corresponding to the C-terminal peptide.

In the case of fraction B, three peaks, 'a', 'b', and 'c', were obtained in this region (Figure 8B). The positions of peaks 'a' and 'b' were identical to those of peaks 'a' and 'b' in fraction A. In this case, a new peak, peak 'c', eluted between peaks 'a' and 'b'. Sequence analysis showed that peak 'a' is DYEEVGVDSVEGEGEEEGEE, which matches the C-terminal sequence of α1/2, and peak 'b' was found to be DVNAAIATIK, which is also specific for α1/2. Peak 'c', which was not found in the unbound fraction A, was found to be a mixture of two peptides: DYEEVGIDSYEDEDEGEE and DVNAAIAAIK; the former matches the C-terminal sequence of α4, and the latter matches the α4 sequence ($\alpha^{327-336}$) (α1/2 differs from α4 in the amino acid residue at 333, which is a threonine in α1/2 and an alanine in α4). In this context, it should be mentioned that in the sequencing chromatogram signals for 2 amino acid residues were obtained for each of the first 10 cycles. The amino acid residues obtained for the first 10 cycles are: D, Y/V, E/N, E/A, V/A, G/I, I/A, D/A, S/I, Y/K. Only a single sequence was obtained for each of cycles 11−20. The amino acid sequence obtained from the 11th through the 18th cycle is EDEDEGEE, which is the C-terminal sequence of α4. No other combination matched with any known tubulin sequence. Thus, fraction B seems to be a mixture of the nontyrosinated forms of α1/2 and α4. In this case also, the sequencing chromatogram exhibited significantly low signals for the underlined residues.

In the case of the NaCl-eluted fraction C, the two closely positioned peaks 'a' and 'b' were obtained (Figure 8C). In this case, peak 'a' eluted slightly more slowly than peak 'a' in fractions A and B. This peak 'a' was found to have the sequence DYEEVGVDSVEGEGEEEGEEY while peak 'b' had the sequence DVNAAIATIK, which are both specific for α1/2. No sequence corresponding to α4 tubulin was detected by Edman degradation. Thus, fraction C is essentially the tyrosinated form of α1/2. Again, like the other fractions, the sequencing chromatogram exhibited significantly lower signals for the underlined residues.

In the case of the KI-eluted fraction (fraction D), four peaks were obtained. In addition to the two peaks 'a' and 'b' (Figure 8D), other minor peaks were observed corresponding to peaks 'a', 'b', and 'c' in fractions A and B. Two major peaks, 'a' and 'b', were sequenced. Peak 'a' gave the sequence DYEEVGIDSYEDEDEGEEY, which corresponds to the C-terminal sequence of the tyrosinated form of α4 tubulin. Peak 'b' gave the sequence DVNAAIAAIK, which also matches the α4 sequence ($\alpha^{327-336}$). Thus, this fraction is predominantly the tyrosinated form of α4, although it contains minor amounts of fragments which appear to correspond to the nontyrosinated forms of α1/2 and α4.

*Colchicine-Binding Activity of the Tubulin Fractions.* One of the major functional properties of tubulin is to bind the antimitotic alkaloid colchicine (*1*, *2*). The binding is slow but stable, and is associated with an increase in drug fluorescence which can be used to monitor the binding (*41*). The ability of tubulin to bind colchicine is very sensitive to the native structure of tubulin, and the binding activity decays with a half-life of about 4−6 h at 37 °C (*2*, *41*). To test whether fractions A, B, and C retain their native structures, the colchicine-binding pattern was studied. For this, the tubulin fractions (2 μM) were incubated with colchicine (100 μM) at 37 °C for 1 h, and the increase in fluorescence emission was recorded at 437 nm upon excitation of the samples at 380 nm. The amount of bound colchicine was calculated from the fluorescence values for the 1 μM tubulin−colchicine complex as described before (*28*). The

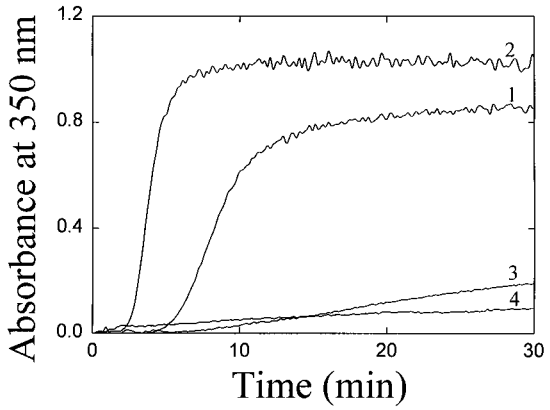


FIGURE 9: Assembly of α-tubulin isoforms in the presence of glycerol and magnesium. Aliquots of tubulin fractions (1 mg/mL) in buffer A containing 16 mM $MgCl_2$ and 4 M glycerol were incubated at 37 °C, and the assembly was studied by monitoring the turbidity of the samples at 350 nm. Different curves are as follows: curve 1, PC−tubulin; curve 2, fraction A; curve 3, fraction B; curve 4, fraction C.

Table 1: Functional Properties of the Different Tubulin Fractions Eluting from the AYN.6D10 Immuonoaffinity Column

| tubulin fractions | elution from column | tyrosination status | α-tubulin class | colchicine binding | assembly |
|---|---|---|---|---|---|
| fraction A | unbound | nontyrosinated | α1/2 | normal | normal |
| fraction B | NaCl peak I | nontyrosinated | α1/2, α4 | normal | poor |
| fraction C | NaCl peak II | tyrosinated | α1/2 | normal | poor |
| fraction D | 3 M KI | tyrosinated | α4 | not tested | not tested |

amounts of bound colchicine were 0.82 ± 0.12, 0.8 ± 0.15, 0.85 ± 0.11, and 0.88 ± 0.12 (moles of colchicine per mole of tubulin dimer) for PC−tubulin, fraction A, fraction B, and fraction C, respectively. The data indicate that the tubulin fractions A, B, and C are functionally active with regard to their colchicine-binding activity.

*Assembly of α-Tubulin Isoforms in the Presence of $Mg^{2+}$.* PC−tubulin is known to assemble in the presence of high $Mg^{2+}$ and glycerol to form polymers resembling normal microtubules (*42*). The isoforms were tested for their assembly activity at a single tubulin concentration in the presence of $Mg^{2+}$ and glycerol. As shown in the figure, the rate and the extent of assembly for the unbound fraction A are little higher than those of the unfractionated PC−tubulin (Figure 9). This small but reproducible increase in assembly for fraction A has always been observed. On the other hand, fractions B and C assembled poorly. The extent of assembly for fractions B and C is found to be about 5−10% as compared to that of unfractionated tubulin. The details of the characterization of different tubulin fractions including their functional properties are summarized in Table 1.

*Copolymerization of Fraction C with the Unbound Fraction.* In an effort to study whether the tubulin from fraction C is capable of assembly, a copolymerization experiment was performed. Here, the unbound fraction was incubated with increasing concentrations of fraction C in the presence of glycerol and magnesium for 30 min, and the microtubules were pelleted on a glycerol cushion at 120000*g* for 5 min in an airfuge. The assembled microtubules were resuspended, boiled with Laemmli sample buffer, and analyzed by SDS−PAGE followed by immunoblotting with the monoclonal antibody AYN.6D10. By virtue of the fact that the antibody recognizes fraction C but not the unbound fraction A, it was

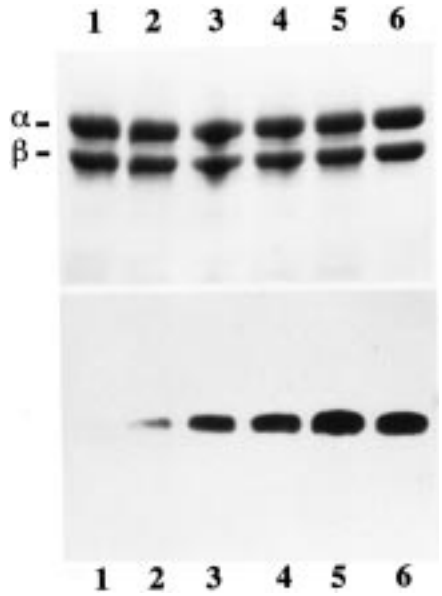


FIGURE 10: Coassembly of fraction C along with fraction A. Aliquots of fraction A (1 mg/mL) in buffer A containing 16 mM $MgCl_2$ and 4 M glycerol were incubated in the presence of different concentrations (0−0.25 mg/mL) of fraction C at 37 °C for 30 min. The microtubules were pelleted by centrifugation on a 50% sucrose cushion in an airfuge. The pellets were resuspended in Laemmli sample buffer and boiled for 5 min before being subjected to SDS−PAGE on 6.5% polyacrylamide gels (upper panel). An identical gel was processed for immunoblotting with the monoclonal antibody AYN.6D10 (lower panel). Since the antibody recognizes fraction C only and not fraction A, the blot demonstrates the ability of fraction C to copolymerize with fraction A. The amount of tubulin loaded in each lane is 5 μg. Fraction C concentrations are: lane 1, 0; lane 2, 0.05 mg/mL; lane 3, 0.10 mg/mL; lane 4, 0.15 mg/mL; lane 5, 0.20 mg/mL; and lane 6, 0.25 mg/mL.

easy to detect the amount of fraction C copolymerized in the pellets. The results show that the amounts of fraction C coassembled with unbound fraction A increased in a concentration-dependent manner (Figure 10). This indicates that although fraction C is unable to assemble by itself, it can copolymerize along with fraction A. That the pelleting of peak C protein was not due to a concentration-dependent aggregation of tubulin in the pellet was confirmed in a control experiment, in which fraction C (at the highest concentration used for the coassembly experiment) was incubated in an identical manner in the absence of fraction A and was centrifuged. No pellet was obtained, indicating that the coassembly of fraction C is not likely due to the concentration-dependent aggregation of tubulin.

## DISCUSSION

The results of the immunoblot analysis of different α-tubulin fusion proteins show that the monoclonal antibody AYN.6D10 recognizes most of the mammalian α-tubulin classes containing a C-terminal tyrosine residue and that antibody recognition is lost upon removal of the C-terminal tyrosine by digestion with carboxypeptidase A. The antibody showed no reactivity with β-tubulin. Thus, the antibody appears to be another anti-tyrosinated tubulin antibody.

Immunoaffinity fractionation of bovine brain tubulin on the AYN.6D10 column yielded four tubulin fractions designated A, B, C, and D, where A is the unbound fraction, B and C are the fractions that eluted with a linear salt gradient,

and D is the fraction that was eluted with 3 M KI after the elution of B and C. Immunoblot analysis of the fractions with mouse anti-Mα4 polyclonal antiserum shows that the NaCl-eluted fraction B and the KI-eluted fraction D contain α4 tubulin, while the unbound fraction A and the NaCl-eluted fraction C do not. Immunoblot results with the antibodies to tyrosinated and nontyrosinated tubulin show that fractions A and B are nontyrosinated whereas fractions C and D are tyrosinated.

Reversed-phase HPLC profile of the endoproteinase Lys-C digest clearly shows that the unbound fraction A contains two sequences which are both specific for α1/2. No other sequence was found to contain either the C-terminal peptide or any other peptide corresponding to α4 tubulin. Immunoblot analysis shows that this fraction reacts only with the monoclonal antibody to nontyrosinated tubulin but not at all with the antibodies to tyrosinated tubulin. Thus, fraction A is essentially the nontyrosinated forms of α1/2 tubulin, that include both the Glu form as well as the Δ2 form. At this time it is not clear about the contribution of the Δ2 form in this fraction; however, the low signal for the $Glu^{450}$ in the sequencing chromatogram may indicate that fraction A may be enriched in the Δ2 form of α1/2. Sequence analysis of fraction B shows that it is a mixture of the nontyrosinated forms of α1/2 and α4. The presence of α4 in this fraction was also confirmed by immunoblotting with antiserum specific for α4 tubulin. It is not known whether this fraction contains any Δ2 tubulin. The analysis of fraction C exhibited two sequences, which are both specific for α1/2. Immunoblotting with antiserum specific for α4 tubulin also confirmed that this fraction is depleted of α4. Thus, fraction C seems to be primarily the tyrosinated form of α1/2 tubulin. Fraction D seems to contain mainly the tyrosinated forms of α4 tubulin. However, it is not clear whether it contains α4 exclusively. Immunoblot results also indicate this. Since this fraction is not functionally active, no further analysis was performed.

It should be mentioned here that the sequencing chromatogram exhibited significantly low signals for some of the amino acid residues in the C-terminal sequences. These residues include the glutamic acid residues $Glu^{445}$, $Glu^{447}$, $Glu^{449}$, and $Glu^{450}$ for α1/2 and $Glu^{445}$, $Glu^{447}$, and $Glu^{448}$ for α4. Usually a low signal can be due to the posttranslational modification of a residue; the modified amino acid residue does not show up in the sequencing chromatogram. It is not clear at this point whether the low signal is due to the posttranslational modification of the residue or is just a sequencing error. It is known that $Glu^{445}$ is modified by posttranslational polyglutamylation, while $Glu^{450}$ (for α1/2) and $Glu^{448}$ (for α4) can be deglutamylated to form the corresponding Δ2 form. Thus, a low signal for $Glu^{445}$, $Glu^{449}$ (in the case of α1/2), and $Glu^{445}$ and $Glu^{447}$ (in the case of α4) can be caused by the covalent modification, while a low signal for $Glu^{450}$ (in the case of α1/2) and $Glu^{448}$ (in the case of α4) can be due to the formation of the corresponding Δ2 form. Extensive mass spectrometric studies are necessary to determine the posttranslational modifications in the tubulin fractions.

It should be mentioned that although the monoclonal antibody AYN.6D10 is specific for the peptide EEGEEY, it exhibits different affinities for different α-tubulin isoforms when used in an immunoaffinity column. The reason is that the specificity of an antibody is determined by Western blotting where the antibody is used at a very high dilution (1/5000 in this case). In contrast, the concentration of antibody in the column is 2−5 mg/mL, which is about 2000-fold higher than the concentration used for Western blotting. Due to this high concentration of the antibody in the immunoaffinity column, a very weak nonspecific affinity for detyrosinated tubulin is observed. Thus, although the epitope for the antibody is the C-terminal tyrosine residue, other factors, such as posttranslational polyglutamylation and/or polyglycylation, that may affect the conformation at the C-terminal region, may also be responsible for the affinity differences. Although it is not clear yet, it seems that the conformation of the COOH-terminal domain for these α-tubulin species may be different, and the affinity of the antibody decreases according to the order: α4-EDEGEEY > α1/2-EEEGEEY > α4-EDEGEE/α1/2-EEEGEE.

The results shown here are quite intriguing in the sense that one can purify the α-tubulin isoforms in the functional state using one immunoaffinity column. The isotypically pure α-tubulin isoforms may be useful for studying microtubule dynamics in vivo. They may also be useful for studying whether they differ in their interactions with motility-related proteins such as dynein and kinesin, or the enzymes responsible for the posttranslational modifications in α-tubulin such as tyrosination, detyrosination, polyglutamylation, and polyglycylation.

The results of the assembly experiment clearly show that unbound fraction A undergoes assembly in the presence of glycerol and $Mg^{2+}$, as does the unfractionated tubulin. In fact, unbound fraction A assembles slightly better (approximately 10−15%) than unfractionated PC−tubulin. Although this difference in assembly is quite insignificant, it has been found to be reproducible in five different assembly studies using different protein preparations. However, a completely different scenario emerged in the case of NaCl-eluted fractions B and C. Very little assembly is observed in 30 min of incubation.

That fractions B and C are not the denatured fractions of tubulin is confirmed by the fact that they retain the colchicine-binding activity, and also can assemble in the presence of 10 $\mu$M taxol to form morphologically normal microtubules (*45*). Furthermore, fraction C can undergo copolymerization with fraction A to form normal microtubules.

The differences in the kinetics of assembly for fractions A, B, and C in the presence of $Mg^{2+}$ may indicate either (a) the isoforms may differ in their interactions with magnesium or (b) different isoforms may differ in their conformational changes upon binding to $Mg^{2+}$ or, in the critical concentration for assembly. Whether the difference in assembly is at the nucleation phase or at the elongation phase remains to be seen.

Although it not known whether the tubulin fractions differ in their polyglutamylation or polyglycylation status, these modifications can certainly affect the assembly property of tubulin. The addition of the glutamyl units as a side chain will certainly increase the net charge at the C-terminal region, while the addition of the glycyl units may lower the net charge to a small extent. Since $Mg^{2+}$ is known to facilitate assembly by counteracting the net negative charge at the

C-terminal region, polyglutamylation in tubulin may result in decreased assembly while polyglycylation may increase it slightly.

In conclusion, I find that the tyrosinated form of α1/2 differs significantly from its nontyrosinated form in the assembly kinetics in the presence of $Mg^{2+}$. The major part of the nontyrosinated form of α1/2 could assemble by itself (fraction A). However, the tyrosinated form could coassemble along with the detyrosinated form. Such a striking difference in the assembly behavior may indicate that the microtubule assembly in vivo may be regulated by different posttranslational modifications in α-tubulin, which may alter the specific interactions of tubulin with different non-tubulin proteins including microtubule-associated proteins such as MAP1, MAP2, MAP4, and tau. It would be interesting to study whether individual α-tubulin isoforms affect the dynamic behavior of microtubules in vivo. Future studies with these isoforms may shed light on the hitherto unknown roles played by the isoforms and their posttranslational modifications in regulating the microtubule function in vivo.

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## REFERENCES

1. Olmsted, J. B., and Borisy, G. G. (1973) *Annu. Rev. Biochem. 42*, 507−540.
2. Dustin, P. (1982) *Microtubules*, Springer-Verlag, Berlin and New York.
3. Lewis, S. A., and Cowan, N. J. (1990) in *Microtubule Proteins* (Avila, J., Ed.) pp 37−66, CRC Press, Boca Raton, FL.
4. Villasante, A., Wang, D., Dobner, P., Dolph, P., Lewis, S. A., and Cowan, N. J. (1986) *Mol. Cell. Biol. 6*, 2409−2419.
5. Sullivan, K. F., and Cleveland, D. W. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 4327−4331.
6. Hecht, N. B., Distel, R. J., Yelick, P. C., Tanhauser, S. M., Driscoll, C. E., Goldberg, E., and Tung, K. S. K. (1988) *Mol. Cell. Biol. 8*, 996−1000.
7. Sullivan, K. (1988) *Annu. Rev. Cell Biol. 4*, 687−716.
8. Wang, D., Villasante, A., Lewis, S. A., and Cowan, N. J. (1986) *J. Cell Biol. 103*, 1903−1910.
9. Ludueña, R. F. (1993) *Mol. Biol. Cell 4*, 445−457.
10. Monteiro, M. J., and Cleveland, D. W. (1988) *J. Mol. Biol. 199*, 439−446.
11. Lee, G. N., Cowan, N. J., and Kirschner, M. W. (1988) *Science 239*, 285−288.
12. Lewis, S. A., Wang, D., and Cowan, N. J. (1988) *Science 242*, 936−939.
13. Barra, H. S., Arce, C. A., Rodriguez, J. A., and Caputto, R. (1974) *Biochem. Biophys. Res. Commun. 60*, 1384−1390.
14. Raybin, D., and Flavin, M. (1975) *Biochem. Biophys. Res. Commun. 65*, 1088−1095.
15. L'Hernault, S. W., and Rosenbaum, J. L. (1983) *J. Cell Biol. 97*, 258−263.
16. L'Hernault, S. W., and Rosenbaum, J. L. (1985) *Biochemistry 24*, 463−478.
17. Paturle-Lafanechère, L., Eddé, B., Denoulet, P., Van Dorsselaer, A., Mazarguil, H., Le Caer, J.-P., Wehland, H., and Job, D. (1991) *Biochemistry 30*, 10523−10528.
18. Eddé, B., Rossier, J., Le Caer, J.-P., Desbruyeres, E., Gros, F., and Denoulet, P. (1990) *Science 247*, 83−84.
19. Alexander, J. E., Hunt, D. F., Lee, M. K., Shabanowitz, J., Michel, H., Berlin, S. C., MacDonald, T. M., Sundberg, R. J., Rebhun, L. I., and Frankfurter, A. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 4685−4689.
20. Redeker, V., Le Caer, J.-P., Rossier, J., and Promé, J.-C. (1991) *J. Biol. Chem. 266*, 23461−23466.
21. Redeker, V., Levilliers, N., Schmitter, J.-M., Le Caer, J.-P., Rossier, J., Adoutte, A., and Bré, M.-H. (1994) *Science 266*, 1688−1691.
22. Eipper, B. A. (1974) *J. Biol. Chem. 249*, 1407−1416.
23. Gard, D. J., and Kirschner, M. W. (1985) *J. Cell Biol. 100*, 746−774.
24. Ludueña, R. F., Zimmermann, H., and Little, M. (1988) *FEBS Lett. 230*, 142−146.
25. Banerjee, A., Roach, M. C., Wall, K. A., Lopata, M. A., Cleveland, D. W., and Ludueña, R. F. (1988) *J. Biol. Chem. 263*, 3029−3034.
26. Banerjee, A., Roach, M. C., Trcka, P. P., and Ludueña, R. F. (1990) *J. Biol. Chem. 265*, 1794−1799.
27. Banerjee, A., Roach, M. C., Trcka, P., and Ludueña, R. F. (1992) *J. Biol. Chem. 267*, 5625−5630.
28. Banerjee, A., and Ludueña, R. F. (1992) *J. Biol. Chem. 267*, 13335−13339.
29. Banerjee, A., D'Hoore, A., and Engelborghs, Y. (1994) *J. Biol. Chem. 269*, 10324−10329.
30. Banerjee, A., Engelborghs, Y., D'Hoore, A., and Fitzgerald, T. J. (1997) *Eur. J. Biochem. 246*, 420−424.
31. Banerjee, A. (1997) *Biochem. Biophys. Res. Commun. 231*, 698−700.
32. Panda, D., Miller, H. P., Banerjee, A., Ludueña, R. F., and Wilson, L. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 11358−11362.
33. Gu, W., Lewis, S. A., and Cowan, N. J. (1988) *J. Cell Biol. 106*, 2011−2022.
34. Fellous, A., Francon, J., Lennon, A.-M., and Nunez, J. (1977) *Eur. J. Biochem. 78*, 167−174
35. Laemmli, U. K. (1970) *Nature 227*, 680−685.
36. Crestfield, A. M., Moore, S., and Stein, W. H. (1963) *J. Biol. Chem. 238*, 622−627.
37. Rushbrook, J. I. (1988) in *Macromolecular sequencing and synthesis: selected methods and applications* (Schlesinger, D. H., Ed.) pp 3−6, Alan R. Liss, Inc., New York.
38. Multigner, L., Pignot-Paintrand, P., Saoudi, Y., Job, D., Plessmann, U., Rüdiger, M., and Weber, K. (1996) *Biochemistry* 35, 10862−10871.
39. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem. 193*, 265−275.
40. Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.
41. Bhattacharyya, B., and Wolff, J. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 2627−2631.
42. Lee, J. C., and Timasheff, S. N. (1977) *Biochemistry 16*, 1754−1764.
43. Schiff, P. B., and Horwitz, S. B. (1979) *Nature 277*, 665−667.
44. Schiff, P. B., and Horwitz, S. B. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1561−1565.
45. Banerjee, A., and Kasmala, L T. (1998) *Biochem. Biophys. Res. Commun. 245*, 349−351.

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