Posttranslational Modifications of the C-Terminus of α-Tubulin in Adult Rat Brain: α4 Is Glutamylated at Two Residues†

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ABSTRACT: In adult mammalian brain, the C-terminus of α-tubulin exhibits a high degree of polymorphism due to a combination of four covalent posttranslational modifications: glutamylation, tyrosination, detyrosination, and removal of the penultimate glutamate residue (C-terminal deglutamylation). Glutamylation is the most abundant. To characterize the glutamylation of α-tubulin and its relationship with the other modifications, we developed a chromatographic procedure for purifying α-tubulin C-terminal peptides. The purified peptides were identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF) and amino acid sequencing. In this report, we provide a complete description of the glutamylation of tyrosinated, detyrosinated, and C-terminal deglutamylated isoforms of both α-tubulin isotypes (α1/2 and α4) expressed in adult rat brain. In particular, we describe for the first time the glutamylation of α4. More than 90% of the α-tubulin is glutamylated, and more than 75% of it is nontyrosinated. α4 is more extensively glutamylated than α1/2, containing as many as 11 posttranslationally added glutamate residues. The most abundant α4 isoform is nontyrosinated, containing five posttranslationally added glutamates, whereas the most abundant α1/2 isoforms are nontyrosinated, with only one or two posttranslationally added glutamates. In contrast to α1/2, α4 is glutamylated at two separate residues (Glu-443 and Glu-445) in the sequence ^{431}DYEEVGIDSYED***E***D***E***GEE^{448}. This is the first evidence that glutamylation can occur on two different residues in the same mammalian tubulin isotype.

In higher vertebrates, α-tubulin and *β*-tubulin, the two proteins that form the tubulin heterodimer, are encoded by small multigene families (*1*, *2*). Each gene family encodes a group of highly similar proteins, referred to as isotypes, that differ from each other primarily by amino acid sequences located within the last approximately 15 C-terminal residues (*3*). These extreme C-terminal regions, referred to as the isotype-defining domains, are known to contain sites of interaction for microtubule-associated proteins (MAPs)[1] and have been implicated in divalent cation binding (*4*−*9*). Recent studies have shown that glutamylation is the major posttranslational modification of brain tubulin and that the glutamylation sites are located within the isotype-defining domains of both α- and *β*-tubulins (*10*−*14*). This unusual covalent modification, which appears to be unique to tubulin, involves the formation of a string of glutamate residues linked through the side chain of a glutamate residue in the polypeptide backbone. The glutamate chain can vary in length from one to as many as seven residues. Whereas the first glutamate is added by an obligatory *γ*-carboxyl linkage, it has been shown that the remaining residues in α-tubulin isotypes are added primarily through α-carboxyl linkages (*15*). It has been proposed that glutamylation, which results in an increase in negative charge within the C-terminus, may facilitate MAP binding and also may serve as a buffer for divalent cations (*16*, *17*).

Of the three α-tubulin isotypes (α1, α2, and α4) expressed in adult mammalian brain, only α1 and α2 have been characterized (*10*, *15*, *18*, *19*). They differ by only a single amino acid substitution outside of the isotype-defining domain. Accordingly, these two are treated as a single protein, α1/2. In addition to glutamylation, α1/2 undergoes three other C-terminal posttranslational modifications: detyrosination, retyrosination, and the removal of the penultimate glutamate from detyrosinated subunits (*20*). Detyrosination involves the removal of the C-terminal tyrosine by a tubulin-specific carboxypeptidase (*21*, *22*). In retyrosination, the tyrosine residue is added back by a tubulin-tyrosine ligase (*21*−*23*). The third posttranslational modification excludes α1/2 from the detyrosination−retyrosination cycle by the removal of the penultimate glutamate residue (*20*, *24*). Nontyrosinatable isoforms have been referred to previously as either N-Tyr or Δ-2 isoforms (*20*, *25*). In this report, C-terminal, deglutamylated isoforms are designated by Δ-Glu. In contrast to α1/2, the α4 gene does not code for

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[1] Abbreviations: MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; *m/z*, mass-to-charge ratio; amu, arbitrary mass units; MAPs, microtubule-associated proteins; HPLC, high-performance liquid chromatography; MES, 2-(*N*-morpholino)ethanesulfonic acid; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol; EGTA, ethylene glycol bis(*β*-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride.

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a C-terminal tyrosine, and the translated protein ends with a glutamate residue (*1*). Although α4 can serve as a substrate for tubulin-tyrosine ligase, it is not clear whether the tyrosinated form is present in brain (*26*, *27*).

The objective of this study was to obtain an extensive picture of the C-terminal posttranslational modifications of α1/2 and α4 in adult brain. In particular, we were interested in characterizing the modifications of the neuron-specific α4 isotype. A new chromatographic procedure for purifying the complete pool of α-tubulin C-terminal peptides was developed. After proteolytic digestion of purified tubulin with endoproteinase Lys-C, these peptides were isolated by arginine−Sepharose chromatography. The peptides were further fractionated by reversed-phase HPLC and characterized by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry and amino acid sequencing. The results provide a complete description of the glutamylation of tyrosinated, detyrosinated, and Δ-Glu isoforms of α1/2 and α4. Whereas 90% of the α-tubulin is glutamylated, 75% is nontyrosinated and 25% is tyrosinated. Furthermore, we demonstrate for the first time that α4 is more extensively glutamylated than α1/2. α4 can contain as many as 11 additional glutamate residues. This level of glutamylation is much higher than that observed for any other mammalian α- or *β*-tubulin isotype. The most abundant α4 isoform is nontyrosinated, containing five posttranslationally added glutamates, whereas the most abundant α1/2 isoforms are nontyrosinated, with only one or two posttranslationally added glutamates. In contrast to α1/2, α4 is glutamylated at two separate residues, Glu-443 and Glu-445. This is the first evidence that glutamylation can occur on two different residues in the same mammalian tubulin isotype.

EXPERIMENTAL PROCEDURES

Tubulin Purification. Microtubule protein was obtained from adult rat brains by one cycle of temperature-dependent assembly and disassembly (*28*). The homogenization and assembly buffer was 50 mM PIPES/NaOH (pH 6.9) containing 50 mM NaF, 1 mM Na_3VO_4, 1 mM $MgSO_4$, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, and 1 mM GTP. NaF and vanadate were added to the buffer to inhibit serine, threonine, and tyrosine phosphatase activity. Microtubule pellets were depolymerized in 25 mM MES/NaOH (pH 6.7) containing 1 mM $MgSO_4$, 1 mM EGTA, 1 mM DTT, and 0.1 mM GTP. Tubulin was purified from the clarified, depolymerized microtubule pellets by arginine−Sepharose chromotography (*29*). Protein was loaded onto the column in depolymerization buffer. Microtubule-associated proteins were eluted from the column by a step gradient consisting of 100 and 200 mM MES/NaOH (pH 6.7) containing 1 mM $MgSO_4$ and 1 mM EGTA. The tubulin was eluted from the column with 25 mM MES/NaOH (pH 6.7) containing 1 mM $MgSO_4$, 1 mM EGTA, and 1 M sodium glutamate. Following elution, the tubulin was repolymerized at 37 °C with the addition of 8% DMSO, 1 mM GTP, and 1 mM $MgSO_4$, and the microtubules were sedimented through a 30% sucrose cushion at 100000*g* for 45 min at 37 °C. The pellets were stored at −80 °C.

Proteolytic Digestion. Tubulin, typically 0.5−1.5 mg, was digested with endoproteinase Lys-C (Boehringer Mannheim) at an enzyme:tubulin ratio of 1:100 (w:w) in 25 mM Tris-HCl (pH 8.5) containing 1 mM EDTA at 37 °C for 18 h. Digestion was terminated by boiling the sample for 5 min. Prior to chromatography, the digest mixture was briefly sonicated and clarified by centrifugation at 12000*g* for 2 min.

α-Tubulin C-Terminal Peptide Purification. Endoproteinase Lys-C digestion of brain tubulin produces α-tubulin C-terminal peptides beginning with aspartate at position 431. To isolate these C-terminal peptides, the digestion mixture was loaded onto an arginine−Sepharose column. Typically, approximately 400 μg of a Lys-C/tubulin mixture was loaded onto a 1 mL column. The column was washed as previously described, and the C-terminal peptides were eluted with 25 mM MES/NaOH (pH 6.7) containing 2.5 M NaCl. The peptides were either desalted on a C18 Sep-Pak cartridge prior to mass spectrometric analysis or further separated by reversed-phase HPLC. Desalting was performed by washing the sample with 0.1% trifluoroacetic acid (solvent A) and eluting with 0.1% trifluoroacetic acid in 80% acetonitrile (solvent B). C18 reversed-phase HPLC (5 μm particle size, 220 mm × 2.1 mm column, Vydac) was performed at a flow rate of 200 μL/min. Peptides were eluted with a gradient consisting of 1% solvent B for 10 min, 1 to 10% solvent B for 1 min, and 10 to 50% solvent B for 80 min. Peptide elution was monitored at 214 nm.

MALDI-TOF Mass Spectrometry. Mass spectra were acquired in the linear and negative mode on a MALDI-TOF mass spectrometer (Voyager Elite, Perseptive Biosystems, Inc., Framingham, MA) equipped with a delayed extraction device. Desorption was produced by a nitrogen laser beam (λ = 337 nm, 3 ns wide pulse at 20 Hz) focused on the target with the laser power set just above the desorption threshold. Ions were detected by the dual channel plate linear detector after a flight of 2 m. The delayed extraction time was set at 175 ns. A total of 100−256 shots were averaged for each acquired spectrum. The sample was mixed 1:1 (v:v) with a saturated solution of either sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid, Aldrich) in 30% acetonitrile and 0.1% aqueous TFA or DHB (2,5-dihydroxybenzoic acid, Aldrich) in 0.1% aqueous TFA and analyzed. All the presented spectra were obtained with sinapinic acid as a matrix. In the negative mode, external calibration was performed using a mixture of neurotensin, ACTH clip(18−39), and ACTH clip(7−38) with average *m*/*z* values corresponding to $[M - H]^-$ of 1671.95, 2464.71, and 3658.17, respectively.

Edman Degradation. Peptides were sequenced by automated Edman degradation using a Procise pulsed-liquid protein sequencer (model 794, Perkin-Elmer Applied Biosystems Division, Foster City, CA).

RESULTS

To assist the reader in interpreting the data presented in this paper, Table 1 provides the masses (average mass-to-charge ratio, *m*/*z*, of $[M - H]^-$ ions) for the tyrosinated, detyrosinated, and Δ-Glu C-terminal peptides of α1/2 and α4 containing zero to six posttranslationally added glutamate residues. All of the C-terminal peptides, produced by endoproteinase Lys-C digestion, begin with aspartate at position 431. It should be noted that it is not possible to distinguish by direct MALDI-TOF mass spectrometry between two peptides with the same amino acid composition.

Table 1: Theoretical Average $[M - H]^-$ Masses Corresponding to Different Levels of Glutamylation for Δ-Glu, Detyrosinated, and Tyrosinated C-Terminal Peptides of α1/2 and α4[a]

	C-terminal sequence	number of posttranslationally added glutamates						
		0	1	2	3	4	5	6
α1/2								
Δ-Glu	DYEEVGVDSVEGEGEEEGE	2055.98	2185.10	2314.22	2443.34	2572.46	2701.58	2830.70
detyrosinated	DYEEVGVDSVEGEGEEEGEE	2185.10	2314.22	2443.34	2572.46	2701.58	2830.7	2959.82
tyrosinated	DYEEVGVDSVEGEGEEEGEEY	2348.28	2477.4	2606.52	2735.64	2864.76	2993.88	3123.00
α4								
Δ-Glu	DYEEVGIDSYEDEDEGE	1990.89	2121.01	2250.13	2379.25	2508.37	2637.49	2766.61
detyrosinated	DYEEVGIDSYEDEDEGEE	2121.01	2250.13	2379.25	2508.37	2637.49	2766.61	2895.73
tyrosinated	DYEEVGIDSYEDEDEGEEY	2284.19	2413.31	2542.43	2671.55	2800.67	2929.79	3058.91

[a] The numbers shown in the table represent mass-to-charge ratios (*m*/*z*) of the deprotonated molecular ions, obtained in the negative and linear mode by MALDI-TOF mass spectrometry. This table shows that direct MALDI-TOF analysis does not discriminate between Δ-Glu, glutamylated peptides and detyrosinated, glutamylated peptides belonging to the same isotype. Only the Δ-Glu, nonglutamylated peptide can be identified unambiguously.

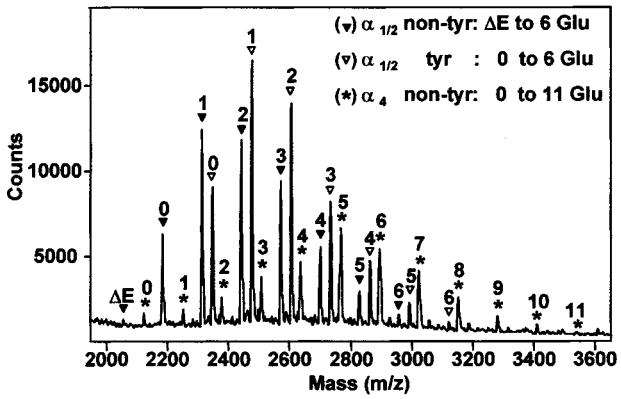

FIGURE 1: MALDI-TOF mass spectrum of the mixture of α-tubulin C-terminal peptides eluted from the arginine−Sepharose column. Mass spectrometric analysis was performed in the linear and negative mode. Three series of molecular ions are observed. They correspond to tyrosinated and nontyrosinated α4 and α2 peptides (open and closed arrowheads, respectively); the series represented by stars corresponds to the nontyrosinated α4 peptides. Each number represents the glutamylation level of the corresponding peptide. The Δ-Glu nonglutamylated α1/2 peptide is denoted ΔE.

Similarly, it is not possible to distinguish between Δ-Glu, glutamylated peptides and detyrosinated, glutamylated peptides belonging to the same isotype. For example, as shown in Table 1, the mass of the Δ-Glu, monoglutamylated α1/2 peptides (m/z = 2185.10) is identical to the mass of the detyrosinated, nonglutamylated α1/2 peptides (m/z = 2185.10). Such discriminations might be possible by fragmentation mass spectrometric analysis.

MALDI-TOF Analysis of the Entire Mixture of α-Tubulin C-Terminal Peptides. In one group of experiments, the pool of arginine−Sepharose-purified peptides, prepared as described in Experimental Procedures, was desalted and analyzed directly by MALDI-TOF mass spectrometry. The conditions of mass spectrometric analysis described in Experimental Procedures were optimized for the analysis of the small C-terminal α-tubulin peptides with masses comprised between 2000 and 4000. The larger C-terminal β-tubulin peptides released by endproteinase Lys-C digestion with masses comprised between 6000 and 8000 were not considered in this study. The mass spectrum obtained for the complex mixture of arginine−Sepharose-purified peptides revealed the presence of a very large number of ions. Three series of ions can be distinguished in Figure 1; each series contains ions which differ from each other by mass increments of 129 amu, the mass of one glutamate residue. The first series, designated by closed arrowheads, begins with a molecular ion (m/z = 2055.44) corresponding to the C-terminal peptide of the Δ-Glu, nonglutamylated α1/2 isotype. The next ion in the series (m/z = 2184.73) could correspond either to a detyrosinated, nonglutamylated α1/2 peptide or to a Δ-Glu, monoglutamylated α1/2 peptide. In reality, this ion most probably corresponds to a mixture of both peptides (*15*). The other five molecular ions in this series represent detyrosinated and Δ-Glu peptides that are more extensively polyglutamylated. The second series of peptides, designated by open arrowheads, begins with a molecular ion (m/z = 2347.89) corresponding to the C terminus of the tyrosinated, nonglutamylated α1/2 peptide. The other ions in this series correspond to the addition of one to six glutamate residues.

A third series of peptides, represented by stars, begins with a molecular ion at m/z of 2120.41 and ends with a molecular ion at m/z of 3540.94. The molecular ions in this series, representing the posttranslational addition from one to 11 glutamate residues, have not been observed previously in mammalian brain tubulin. The 12 peptides were tentatively identified as α4 isoforms. To verify the identity of the glutamylated peptides in the third series of ions, arginine−Sepharose-purified α-tubulin C-terminal peptides were further fractionated by reversed-phase HPLC.

Three major fractions were obtained by C18 reversed-phase HPLC (Figure 2). These three fractions correspond to the three series of peptides observed in the MALDI-TOF analysis of the complex peptide mixture. The peptides contained in each of the three HPLC fractions were characterized by both mass spectrometry and amino acid sequencing. Amino acid sequencing by Edman degradation provides the exact amino acid sequence of the peptide and the location of the posttranslationally modified residue(s). As reported previously for glutamylation, glutamylated residues are not identified by Edman degradation chemistry. The very polar derivatized glutamylated amino acid is not released from the filter and thus appears as a "gap" in the sequence (*10*, *11*).

Identification of Glutamylated α4 Tubulin C-Terminal Peptides. Amino acid sequencing of the peptides contained in HPLC fraction 2 yielded the sequence DYEEVGIDSYEDXDXG. This sequence matches the C-terminal sequence of α4 tubulin from residue 431 to residue 446. The Xs at positions 443 and 445 indicate that the encoded

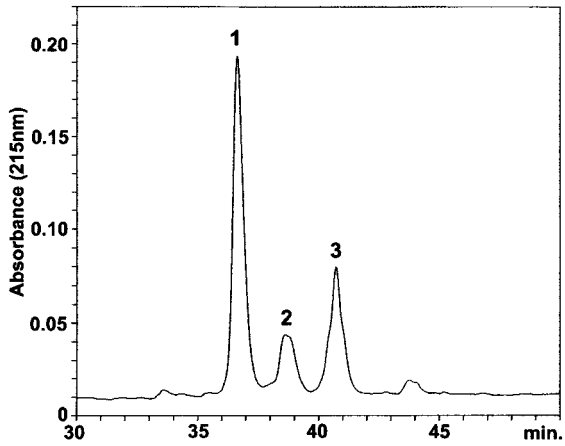

FIGURE 2: Reversed-phase HPLC spectrum of arginine−Sepharose-purified α-tubulin peptides. α-Tubulin C-terminal peptides obtained from the purification of 390 μg of digested tubulin were separated on a C18 column. Three major peaks (1−3) were resolved. The peptides contained in each peak were characterized by MALDI-TOF mass spectrometry and Edman degradation amino acid sequencing.

glutamate residues at these positions are posttranslationally modified. This is the first evidence of two separate glutamylation sites for one brain tubulin isotype. To determine the glutamylation level of the α4 peptides contained in fraction 2, they were characterized by MALDI-TOF. Twelve molecular ions are present in Figure 3A, revealing a maximum of 11 additional glutamates. The first ion (*m*/*z* ratio = 2121.02) represents a peptide with the sequence ^{431}DYEEVGIDSYEDEDEGE(E)^{448}, corresponding to either the detyrosinated, nonglutamylated α4 C-terminus or the Δ-Glu, monoglutamylated α4 C-terminus. In this experiment, an ion matching the mass of the Δ-Glu, nonglutamylated peptide was not unequivocally identified, suggesting that α4 Δ-Glu peptides are minor constituents of the peptide pool. Of the 12 α4 peptides represented in Figure 3A, the most abundant are those containing four, five, and six posttranslationally added glutamate residues.

Mass spectrometric analysis of a very minor HPLC fraction revealed the presence of multiple ions with masses matching those of glutamylated, tyrosinated α4 peptides (data not presented). This observation suggests that trace quantities of tyrosinated α4 isoforms are present in adult rat brain. Since we were unable to verify the identity of this isoform by amino acid sequencing, these very low-abundance peptides were not included in the estimates discussed in the following sections.

Identification of Glutamylated α1/2 Tubulin C-Terminal Peptides. Amino acid sequencing of the peptides contained in HPLC fraction 1 yielded the sequence DYEEVGVDSVEGEGXEEG. This sequence matches the C-terminal sequence of α1/2 tubulin from residue 431 to residue 448. The X at position 445 indicates that the corresponding glutamate residue is posttranslationally modified. To determine more precisely the posttranslational modifications of the α1/2 peptides present in fraction 1, they were identified by MALDI-TOF. Ten molecular ions are present in Figure 3B, revealing a maximum of eight additional glutamates. The first ion (*m*/*z* = 2055.59) represents a peptide with the sequence ^{431}DYEEVGVDSVEGEGEEEGE^{449}, corresponding to the Δ-Glu, nonglutamylated C-terminus of α1/2. The

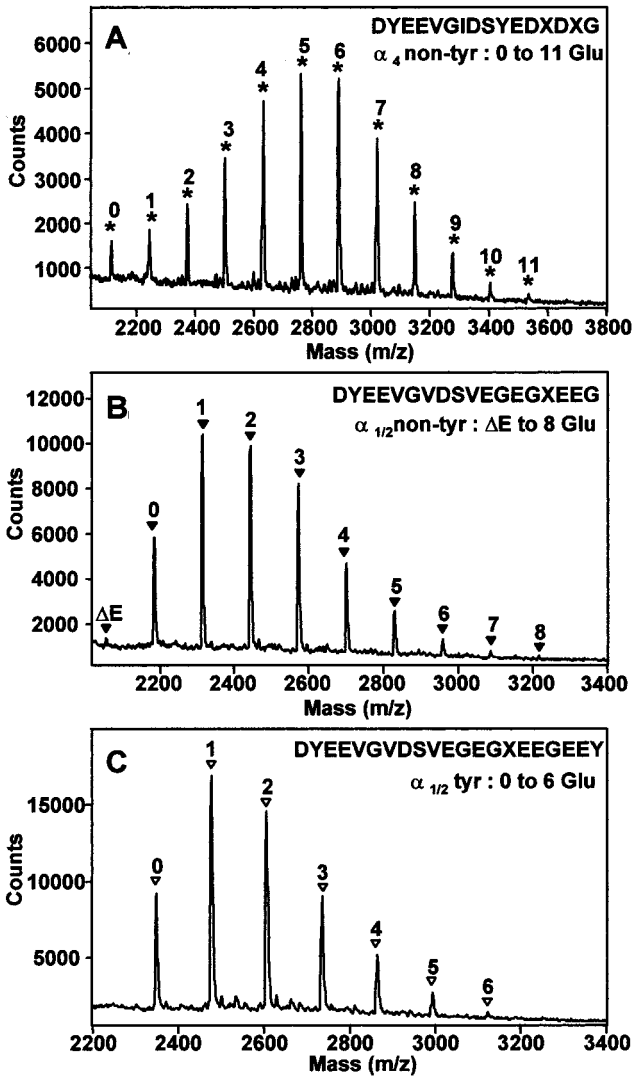

FIGURE 3: MALDI-TOF mass spectra of the three reversed-phase purified fractions. The peptides contained in HPLC fractions 1−3 were first sequenced by Edman degradation chemistry and then characterized by MALDI-TOF mass spectrometry analysis in the linear and negative mode. The sequence of the peptides in each fraction is presented. (A) The mass spectrum of nontyrosinated α4 peptides present in fraction 2 shows a series of 12 ions, separated by increments of 129 amu. The number of posttranslationally added glutamate residues is indicated above each ion. The spectrum demonstrates that nontyrosinated α4 can be modified by the addition of as many as 11 glutamates. (B) The mass spectrum of nontyrosinated α1/2 peptides present in fraction 1 shows a series of 10 ions, separated by increments of 129 amu. The spectrum demonstrates that nontyrosinated α1/2 can be modified by the addition of as many as eight glutamates. (C) The mass spectrum of tyrosinated α1/2 peptides present in fraction 3 shows a series of seven ions, separated by increments of 129 amu. The spectrum demonstrates that tyrosinated α1/2 can be modified by the addition of as many as six glutamates.

other nine ions represent mixtures of detyrosinated or Δ-Glu α1/2 peptides. For example, the second ion in the series (*m*/*z* = 2184.86) corresponds in mass to both the detyrosinated, nonglutamylated peptide and the Δ-Glu, monoglutamylated peptide. Of the 10 α1/2 peptides represented in Figure 3B, the most abundant are either detyrosinated or Δ-Glu peptides containing either one or two, or two or three, additional glutamate residues, respectively. The maximum level of α1/2 glutamylation shown in Figure 3B is greater than that shown in Figure 1. The increased sensitivity is

Table 2: Relative Abundance, Glutamylation Sites, and Glutamylation Level of α1/2 and α4 in Adult Rat Brain[a]

isotype	sequence	relative abundance (%)	maximum no. of Glu	most abundant
α1/2	^{431}DYEEVGVDSVEGEG**E**^{445}EEGEEY^{451}	25	6	1 or 2 Glu
α1/2	^{431}DYEEVGVDSVEGEG**E**^{445}EEGE^{449}(E^{450})	60	8	1 Glu
α4	^{431}DYEEVG I DSYED**E**^{443}D**E**^{445}GEE^{448}	15	11	5 or 6 Glu

[a] The locations of the glutamylated residues are bold. Of the α-tubulin isoforms identified, the relative abundance of each isotype was calculated as described in the Results.

due to better ionization and desorption of the purified α1/2 peptides. In the complex peptide mixture, ion suppression is observed for some low-abundance peptides. This phenomenon is reported as spectral suppression (*30*).

The amino acid sequence of the peptides in fraction 3 was DYEEVGVDSVEGEGXEEGEEY. This sequence corresponds to the C-terminus of tyrosinated α1/2 from residue 431 to the C-terminal tyrosine residue at position 451. In contrast to the nontyrosinated peptides, in which the two C-terminal amino acids, hydrophilic glutamate residues, are not detected by Edman sequencing, the last tyrosine, a hydrophobic residue, was clearly identified. A series of seven ions were observed by MALDI-TOF analysis of fraction 3. The first ion (m/z = 2348.22) corresponds to the tyrosinated, nonglutamylated C-terminal peptide of α1/2 tubulin. The following ions in this series correspond to the addition of one to six glutamate residues. The predominant peptide in this series of tyrosinated α1/2 peptides is that with one additional glutamate residue.

Relative Abundance of α1/2 and α4 Isoforms. To obtain an estimate of the relative abundance of each series of C-terminal peptides described in the preceding section, we calculated the area under the peak for each of the reversed-phase HPLC fractions. The relative abundance of each group of peptides was estimated by dividing the area of each fraction by the sum of the areas of the three HPLC fractions. Three separate reversed-phase chromatograms were used to obtain the estimates presented in Table 2. Of the α-tubulin isoforms identified in this study, 85% are α1/2 variants and 15% are α4 variants. As previously explained, no distinction can be made between the detyrosinated and Δ-Glu peptides from each isotype. Accurate estimates of the relative abundance of these peptides cannot be obtained from the intensities (counts) of the molecular ions shown in the mass spectra. Using synthetic peptides, we determined that tyrosinated peptides desorb more efficiently than detyrosinated peptides by a factor of 2 (data not presented). However, within a series containing the same peptide backbone (linear sequence), the level of glutamylation has only a negligible effect on desorption. Consequently, the mass spectra can be used to estimate the relative abundance of differently glutamylated species within the same series of molecular ions.

DISCUSSION

In this study, we describe a chromatographic procedure for purifying the entire pool of α-tubulin C-terminal peptides from adult rat brain tubulin. Tubulin was first purified by arginine−Sepharose chromatography. The purified tubulin was proteolytically digested, and the α-tubulin C-terminal peptides were further separated on arginine−Sepharose followed by reversed-phase HPLC. Amino acid sequencing and mass spectrometry of the purified peptides provided an extensive profile of the many posttranslationally modified α-tubulin isoforms in adult rat brain.

We estimate that α1/2 isoforms constitute approximately 85% of the α-tubulin subunit pool. Of the α1/2 isoforms, approximately 30% are tyrosinated and 70% are nontyrosinated. As previously explained, we are unable to determine the relative abundance of detyrosinated and Δ-Glu isoforms within the nontyrosinated pool. For both tyrosinated and nontyrosinated α1/2 isoforms, the most abundant variants contain one or two posttranslationally added glutamates. We further estimate that glutamylated isoforms constitute approximately 85−90% of the α1/2 isotype pool.

The remaining 15% of the α-tubulin pool is composed of α4 isoforms. As discussed in the introductory section, the translated α4 protein ends with a glutamate residue because the gene does not encode a C-terminal tyrosine (*1*). Although α4 can serve as a substrate for tubulin-tyrosine ligase, the tyrosinated isoform is not detectable in Western blots of adult rat brain extracts (*26*, *27*). However, this isoform can be detected by immunocytochemistry (*27*). Consistent with these observations, the α4 peptides that we identified by both mass spectrometry and amino acid sequencing were nontyrosinated. However, we were able to detect trace levels of tyrosinated α4 by mass spectrometry only. Of the nontyrosinated α4, approximately 95% is glutamylated. This isotype can contain as many as 11 posttranslationally added glutamates, a level of glutamylation which is considerably higher than that observed for α1/2. Moreover, the most abundant polyglutamylated α4 isoform possesses five additional residues, whereas the most abundant glutamylated α1/2 isoform contains only one additional glutamate. In *Trypanosoma brucei*, α-tubulin isoforms in the subpellicular and flagellar microtubules can possess as many as 15 additional glutamates (*31*). However, in *T. brucei*, the most abundant glutamylated isoform contains only one posttranslationnally added glutamate residue. Finally, we have definitively demonstrated that α4 is glutamylated at two different glutamate residues (Glu-443 and Glu-445). This is the first evidence for multiple-site glutamylation for any mammalian tubulin isotype. What remains to be determined is whether both modification sites are occupied in the same polypeptide. In *Paramecium*, mass spectrometric fragmentation studies have shown that glycylation of tubulin can be distributed on four different sites within the same molecule (*32*). Similarly, a multisite distribution within the same molecule could be expected for α4 glutamylation. Further fragmentation studies are necessary to resolve this question.

Sequence alignment of the tubulins known to be glutamylated reveals that neither of the two α4 glutamylation sites aligns with the position of the glutamylation sites in any of the other mammalian α-tubulins (Table 3). Instead of the motif GEG*E*EE where the underlined glutamate residue is glutamylated in α1/2, α4 contains a more acidic motif

Table 3: Sequence Alignment of the Glutamylation Sites for All of the α- and β- Tubulin Isotypes Expressed in Adult Rat Brain Tubulin and in *T. brucei*[a]

Subunit	Isotypes	Sequences	
α	α 1/2	DYEEVGVDSV EGEG**E**EEGEEY	*35*
	α 1/2	DYEEVGVDSV EGEG**E**EEGE(E)	*10*
	α 4	DYEEVG I DSYE - D -**E**D**E**GEE	*This work*
		or DYEEVG I DSYE D**E**D**E**GEE	
β	β I	DATAEE EE DFGEEA**E**EEA	*14*
	β II	DATAD - - - - - - EQG**E**FEEEEGEDEA	*12, 13*
	β III	DATAEE E - - -GEMY**E**DDDEESEAQGPK	*11*
	β IVa	DATAE - - - - - - E -G**E**FEEEAEEEVA	*14*
α	Tryp	AE - - - SADMDG**E**EDVEEY	*31*
β	Tryp	DAT IE - - - - - E - EG**E**FDEEEQY	*31*

[a] The locations of the glutamylated residues are bold and underlined.

YED**E**D**E**GEE. Furthermore, unlike the other α-tubulins, in α4 an aromatic residue is close to the glutamylation sites. An inspection of the glutamylation sites in β-tubulins also reveals the presence of an aromatic residue (tyrosine or phenylalanine) close to their glutamylation sites. The comparison of the glutamylation sites that have been precisely characterized for the β- and the α-tubulins is shown in Table 3. It appears that the glutamylating enzyme(s) does not require a strictly conserved sequence motif, although specific glutamate residues are modified in each isotype. The existence of several glutamylating enzymes with high specificities could explain the site selectivity associated with glutamylation. Recent data about tubulin polyglutamylase are in accordance with this hypothesis (*33*).

Does α4 possess functional properties different than those of α1/2? The fact that it is substantially more glutamylated than α1/2, contains two glutamylation sites rather than one, and is almost exclusively present in the nontyrosinated form suggests that it should. In its extensively glutamylated state, α4 may enhance the binding of MAPs and may facilitate the chelatation of Ca^{2+} ions. In addition to being expressed in brain, in which it is apparently expressed exclusively in neurons (*27*), α4 is expressed in a wide variety of tissues but is most abundant in striated, smooth, and cardiac muscle (*1*). Calcium homeostasis is an important aspect of muscle metabolism. Determining the extent to which α4 is modified in muscle, as well as in other tissues, will be informative.

With respect to MAP binding, does α4 interact differently with MAPs than α1/2? We have demonstrated in this paper that the most abundant α4 isoform contains five posttranslationally added glutamates and that the most abundant α1/2 isoforms contain one or two additional glutamates. Two recent blot-overlay studies have shown that the efficiency of MAP−tubulin interactions varies with the length of the lateral glutamate chain (*16*, *17*). This interaction can be represented as an inverted U-shaped function, in which short and long lateral glutamate chains decrease the efficiency of MAP binding. It was reported that the efficiency of MAP binding was optimal for three posttranslationally added glutamates. In this regard, what effect the glutamylation level of each isotype might have on MAP binding and microtubule dynamics is unclear. This issue might be partially resolved by comparing the assembly properties of immunoaffinity-purified α4 and β-tubulin subunits with those of α1/2 and β-tubulin subunits and with unfractionated tubulin, in the presence and absence of MAPs. Luduena and co-workers have utilized this approach to study the microtubule dynamics of affinity-purified β-tubulin isotypes and their associated α-tubulin subunits (*34*). Ultimately, the function of glutamylation will be better understood only when the glutamylating enzyme(s) and deglutamylating hydrolases will be isolated.

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