Coordination of Posttranslational Modifications of Bovine Brain α-Tubulin

POLYGLYCYLATION OF Δ2 TUBULIN*

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Microtubules participate in a large number of intracellular events including cell division, intracellular transport and secretion, axonal transport, and maintenance of cell morphology. They are composed of tubulin, a heterodimeric protein, consisting of two similar polypeptides α and β. In mammalian cells, both α- and β-tubulin occur as seven to eight different genetic variants, which also undergo numerous posttranslational modifications that include tyrosination-de tyrosination and de glutamylation, phosphorylation, acetylation, poly glutamylation, and polyglycylation. Tyrosination-de tyrosination is one of the major posttranslational modifications in which the C-terminal tyrosine residue in α-tubulin is added or removed reversibly. Although this modification does not alter the assembly activity of tubulin in vitro, these two forms of tubulin have been found to be distributed differently in vivo and are also correlated with microtubule stability (Gunderson, G. G., Kalnoski, M. H., and Bulinski, J. C. (1984) Cell 38, 779–789). Thus, the question arises as to whether these two forms of tubulin differ in any other modifications. In an effort to answer this question, the tyrosinated and the nontyrosinated forms of the α1/2 isofrom have been purified from brain tubulin by immunoaffinity chromatography, matrix-assisted laser desorption/ionization-time of flight mass spectrometric analysis of the C-terminal peptide revealed that the tyrosinated form is poly glutamylation with one to four Glu residues, while the Δ2 tubulin is polyglycylated with one to three Gly residues. These results indicate that posttranslational modifications of tubulin are correlated with each other and that poly glutamylation and polyglycylation of tubulin may have important roles in regulating microtubule assembly, stability, and function in vivo.

Microtubules are eukaryotic structures that maintain cell morphology and mediate diverse and vital cellular functions including mitosis, intracellular transport, and secretion (1–4). Tubulin, the heterodimeric αβ subunit of microtubules, exists as multiple isoforms whose expression patterns differ among tissues (5–23). In mammalian system, there are at least seven different isoforms of α-tubulin designated as α1, α2, α3/7, α4, α6, α8, and αTT1 (18-23). On the other hand, eight β-tubulins are designated as β1, βII, βIV, βVNA, βVNB, βVII, and βVIII. Whether the functional diversity of microtubules arises from the existence of different tubulin isoforms is not very clear. However, in vitro studies have demonstrated that tubulin heterodimers containing different β-tubulins have significant differences in their assembly (23), drug-binding (24–28), conformation (29, 30), and stability (31) as well as in the dynamics of microtubules they form (32, 33).

In addition to the existence of different genetic variations, α- and β-tubulin also undergo a number of covalent modifications that include tyrosination-de tyrosination (34–42) and de glutamylation of the α-tubulin C terminus or the formation of Δ2 tubulin (α-tubulin lacking both the Glu and the Tyr residues from the C terminus) (43) and acetylation at Lys\(^{12}\) (44, 45); \(β_{II}\)-tubulin undergoes phosphorylation at a serine residue (46); both α- and β-tubulin also undergo poly glutamylation and polyglycylation, in which glutamyl or glycyl units are attached as side chains through the γ-carboxyl of a Glu residue near the C terminus (46–56). Poly glutamylation of tubulin has hitherto been observed in flagellar and ciliary microtubules (48), and could conceivably play a role in the unusual stability and morphology of these microtubules. Except for acetylation and phosphorylation, most of these covalent modifications occur near the C termini of α- and β-tubulin.

It is not known exactly how different posttranslational modifications affect the function of tubulin and microtubules in vivo. Although tyrosinated and nontyrosinated tubulin do not appear to differ in the assembly (36, 40), these two forms of tubulin are known to be distributed differently in the interphase microtubules and are believed to form separate populations of microtubules. Microtubules enriched in de tyrosinated tubulin appear to be much more stable than those enriched in tyrosinated tubulin (42). Thus, it seems possible, that the tyrosinated and the nontyrosinated forms of tubulin may differ in other covalent modifications that may give rise to different subsets of microtubules in vivo.

In an effort to study how different covalent modifications may contribute to the structure and function of a tubulin isoform, tubulin dimers were separated on an immunoaffinity column that contained a monoclonal antibody to tyrosinated 1/2 tubulin (56). My previous results demonstrated that the tubulin dimers containing tyrosinated form of 1/2 assemble poorly in the presence of glycerol and Mg\(^{2+}\). In this manuscript, I have analyzed the covalent modifications of tyrosinated and nontyrosinated 1/2 by MALDI-TOF\(^{3}\) mass spectrometry. The

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1 The abbreviations used are: MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; HPLC, high performance liquid chromatography; Mes, 2-(N-morpholinol)ethanesulfonic acid; MAP, microtubule-associated protein.
results show that tyrosinated α/2 is polyglutamylated with one to four Glu residues, the tetraglutamylated form being the predominant one. On the other hand, the Δ2 form of α/2 that assembles normally is polyglycylated with one to three glycyl units, the biglycylated form being the major one. These results indicate that there is a correlation between tyrosination/detyrosination and deglutamylation of tubulin on one hand and polyglycylatation/polyglutamylation on the other hand. This correlation among posttranslational modifications of tubulin is a novel finding. Since tyrosination/detyrosination appears to play a role in microtubule stability, these results raise the possibility that polyglucylation and polyglutamylation may also be involved in microtubule stability and function. These results also indicate that polyglycylatation is not restricted to tubulin destined to form axonal microtubules.

EXPERIMENTAL PROCEDURES

MATERIALS—Mes, CNBr-activated Sepharose, trifluoroacetic acid, and a-cyano-4-hydroxycinnamic acid were obtained from Sigma. Bovine brain cortex was obtained from Kiolbassa Meat Company, San Antonio, TX.

Preparation of Tubulin and Microtubule-associated Proteins—Microtubules were isolated from bovine brain cortex by one cycle of assembly and disassembly as described before (17). Tubulin was purified from microtubules by phosphocellulose chromatography. MAP2 and tau were purified from microtubule proteins by ultrogel chromatography. All purifications were carried out in Mes-Na (pH 6.4), 1 mM EGTA, 0.1 mM EDTA, 0.5 mM MgCl2, and 1.0 mM GTP. Preparations of α-Tubulin—Microtubules were pelleted by centrifugation at 17,000 × g and resuspended in 8 M glycerol at a concentration of 5 mg/ml. Samples were sonicated, and the resulting slurry containing microtubules was filtered through a 0.2 μm membrane. An aliquot of the protein sample was used to prepare SDS-PAGE samples to determine the concentration of α-tubulin. Microtubules were then pelleted by centrifugation at 17,000 × g for 30 min and resuspended in 8 M glycerol in a volume of 0.5 ml. The samples were filtered through a 0.2 μm membrane and centrifuged at 20,000 × g for 30 min. The resulting supernatant was divided into aliquots and stored at −80 °C or at −20 °C.

Preparation of α-Tubulin Isoforms—Bovine brain tubulin contains two α-tubulin isoforms, α1/2 and α4. Using an anti-α-tubulin immunoaffinity column, we have previously fractionated bovine brain tubulin into three functionally active forms, fractions A, B, and C (56). Immunoblot analysis showed that this fraction is not recognized by the monoclonal antibodies to tyrosinated tubulin. Sequence analyses have shown that Fraction A contains nonpolyglutamylated forms of α1/2 that may include Δ2 tubulin. Fraction B is a mixture of the nonpolyglutamylated forms of α1/2 and α4. Fraction C is essentially the tyrosinated form of α1/2. Since fractions A and C are primarily the nonpolyglutamylated and the tyrosinated forms of α1/2, these constitute an appropriate system for structural comparisons. Thus, it was interesting to study the posttranslational modifications on these two fractions by MALDI-TOF analyses.

Mass Spectrometric Analysis of the C-terminal Peptides of the α-Tubulin Isoforms—Bovine brain tubulin contains two α-tubulin isoforms, α1/2 and α4, and each of the isoforms have three posttranslational variations, namely the tyrosinated (Tyr form), the detyrosinated (Glu form), and the Δ2 form (lacking both the Tyr and Glu residues from the C terminus). The expected m/z values for the protonated forms (MH+) are given in Table I.

To study the posttranslational status of the purified tubulin fractions, the C-terminal peptide was isolated by preparative SDS-PAGE, followed by digestion with endoproteinase Lys-C as previously described (56). The C-terminal peptide was separated by reversed-phase HPLC on a C18 column and was identified by sequencing the N-terminal five residues (DYEEV) since this sequence does not exist anywhere other than the C-terminal. The fractions that were confirmed to have the C-terminal sequence were examined by MALDI-TOF.

Fig. 1A shows the HPLC profile of the endoproteinase Lys-C-digested Fraction A. Sequence analysis shows that the C-terminal peptide is present in two peaks: a major peak a followed by a minor peak b (A). The peaks a and b were both found to contain the sequence DYEEVGDSVEGEFEGEE, which corresponds to the Δ2 form of α1/2. The underlining of the Glu residues (Glu^40, Glu^47, and Glu^55) signifies that signals for those residues in the sequencing chromatogram were poor, indicating that the residues might be modified by posttranslational modification. MALDI-TOF analysis revealed that peak a exhibits a single major peak at m/z value of 2170 and several minor peaks at 2227.8, 2322.66, 2387.29, 2451.7, and 2516.22 (Fig. 1C). Peak b exhibits peaks at 2113.82, 2149, 2170, and 2227.8. The peaks at 2113 and 2170 differ by 57, which is the mass value of a glycyl unit (2170−2113 = 57), and the peaks at

| Tubulin isoform | Sequence | Expected mass values for the MH⁺ in MALDI-TOF |
|----------------|----------|-----------------------------------------------|
| Δ2-α/2         | DYEEVGSYSEDEDEGE | 2056.81                                       |
| Glu-α/2        | DYEEVGSYSEDEDEEEG | 2285.84                                       |
| Tyr-α/2        | DYEEVGSYSEDEDEGE | 2185.84                                       |
| Δ2-α1/2        | DYEEVGSYSEDEDEEG | 2056.81                                       |
| Glu-α1/2       | DYEEVGSYSEDEDEEGE | 2185.84                                       |
| Tyr-α1/2       | DYEEVGSYSEDEDEEEGG | 2348.89                                       |
2170 and 2227 also differ by 57 (2227 – 2170 = 57). Since the mass values for the unmodified Δ2 forms for α1/2 and α4 are 2057 and 1993, respectively, the peaks at 2113, 2170, and 2227 originate by the addition of 1, 2, and 3 glycylic units to the Δ2 form of α1/2. Thus Fraction A is modified with one to three posttranslationally added glycylic residues, the biglycyclated being the major species.

At this point, it is not possible to identify the three minor peaks at 2149, 2323, and 2387. The peak at 2149 may have originated from the Δ2 form of α1/2 or α4. If it originates from α1/2, the mass of the modification would be 92 (2149 – 2057 = 92). No known modification to this date exists with this mass value. On the other hand, if it originates from α4, the mass of the modification would be 156 (2149 – 1993 = 156), which is the mass of an arginine residue. At this point it is difficult to conclude about this modification. The mass peaks at 2323 and 2387 are 200 mass units higher than the expected mass values for the Glu forms for α4 and α1/2, respectively, (2123 + 200 = 2323, 2187 + 200 = 2387). The other minor peaks at 2451 and 2516 seems to be derived from 2323 and 2387 by the addition of a mass unit of 129, which is a Glu residue (2452 = 2323 + 129, 2516 = 2387 + 129).

The analysis for tyrosinated α1/2 is summarized in Fig. 2. As shown in Fig. 2A, the C-terminal peptide eluted as a single peak. Sequence analysis showed that this peptide contains the sequence DYEYVDVDSVEGEKEEGEY, which is the sequence of the tyrosinated form of α1/2. Here also the signals for the underlined Glu residues (Glu⁴⁴, Glu⁴⁷, Glu⁴⁹, and

**Fig. 1.** MALDI-TOF analysis of the C-terminal peptide of nontyrosinated tubulin. A, HPLC purification of the C-terminal peptide from nontyrosinated tubulin (unbound fraction). Bovine brain PC-tubulin was passed through an immunosorbent column containing covalently bound anti-α-tubulin monoclonal antibody AYN.6D10. The unbound fraction was pooled and subjected to SDS-PAGE on a preparative gel to separate α- and β-tubulin. The α-tubulin band was cut out from the stained gel, and the protein was electroeluted and purified by acetone precipitation. 15 μg of α-tubulin was digested with endoprotease Lys-C at room temperature for 16 h. The digest was subjected to reversed phase HPLC on a C-18 column. The peaks were sequenced for the first five residues, and the C-terminal peptides were identified. Peaks marked a and b are the C-terminal peptides. B and C are the mass spectrometric analysis of the peaks a and b, respectively. Notice that the major component of peak a has a m/z value of 2170 and a minor component at 2227, peak b, exhibits m/z values of 2113 and 2170. The MALDI peaks marked by 1G, 2G, and 3G correspond to posttranslational addition of one, two, and three glycylic units, respectively, to the Δ2 form of α1/2 tubulin (m/z = 2056).

**Fig. 2.** MALDI-TOF analysis of the C-terminal peptide. A, HPLC purification of the C-terminal peptide from tyrosinated α1/2 tubulin. Bovine brain PC-tubulin was chromatographed on the AYN.6D10 column, tyrosinated α1/2 tubulin was purified as described under "Experimental Procedures," and the α-tubulin was isolated. 15 μg of α-tubulin was digested with endoprotease Lys-C as described before. The digest was subsequently subjected to reversed phase HPLC as done for Fig. 1 and the C-terminal peptide was identified. B shows the MALDI-TOF analysis of the C-terminal peptide. Notice that the major peak at 2866.57 corresponds to the tetraglutamylated form of tyrosinated α1/2 tubulin, while the minor peaks at 2350, 2479, 2608.5, 2737.54 correspond to species formed by sequential addition of zero to three Gly residues to the tyrosinated α1/2 tubulin. The peak at 2350 corresponds to tyrosinated α1/2 with no glycyl residue. The peaks at 2479, 2609, 2737, and 2866 are derived from the addition of one, two, three, and four Glu units (m/z 129) to 2350 (2479 – 2350 + 129, 2608 = 2350 + 2 × 129, 2737 = 2350 + 3 × 129, 2866 = 2350 + 4 × 129). Thus this fraction seems to be polyglutamylylated with one to four Glu residues, and the tetraglutamylated is the major one.
Glutamate residues were poor, indicating that the residues may be modified by posttranslational modification.

MALDI-TOF analysis of this tubulin isoform as shown in Fig. 2B, revealed a major peak at 2866.57 and several minor peaks at 2057, 2350, 2479, 2608.5, and 2737.54. The peak at 2350 corresponds to tyrosinated α1/2, and the peaks at 2479, 2608, 2737, and 2866 appear to be derived from the addition of one, two, three, and four Glu units (m/z 129) to 2350 (2479 = 2350 + 129; 2608 = 2350 + 2 × 129; 2737 = 2350 + 3 × 129; 2866 = 2350 + 4 × 129). Thus this fraction seems to be polyglutamylated with one to four Glu residues, and the tetraglutamylated is the major one.

Amino Acid Analysis—In an effort to confirm the presence of posttranslationally added Gly residues, amino acid analyses were performed on the C-terminal peptides. Since the peptide contains one Ser residue, the amount of Gly residues was calculated per mole of Ser present in the peptide. Analysis of Δ2 tubulin revealed that this fraction (Fraction A) contains 6.2 moles of Gly per mole of Ser. Similar analysis for the tyrosinated tubulin showed that it contained 4 moles of Gly per mole of Ser. These results clearly demonstrate that the Δ2 tubulin is modified with more than two posttranslationally added Gly residues, while the tyrosinated tubulin does not contain any posttranslationally added Gly residue.

DISCUSSION

In my previous study, different α-tubulin isoforms were separated in functionally active forms. The fraction that did not bind the affinity column was found to be the nontyrosinated form of α1/2, while the tyrosinated α1/2 was eluted from the column using a salt gradient. These two fractions exhibited remarkable differences in their assembly properties. The nontyrosinated fraction assembled fairly well, while the tyrosinated fraction did poorly. Thus, it was tempting to study whether these two fractions differ in their posttranslational modifications. In this study, I have studied the posttranslational status of affinity-purified α-tubulin isoforms by MALDI-TOF mass spectrometry.

Mass spectrometric data for the nontyrosinated α1/2 exhibited the major peak at 2170 with some minor peaks at 2113 and 2227. The peaks at 2170 clearly indicate that this must be smaller than the Glu form of α1/2. This fraction must therefore be the Δ2 form of α1/2. The minor peak at 2113 differs from the mass of the Δ2 form by 57. The peaks at 2113, 2170, and 2227 are formed by the addition of one, two, and three glycol units (57 mass units) to the Δ2 form of α1/2. Amino acid analyses of the C-terminal peptide also confirmed the presence of more than two Gly residues per mole of the peptide.

The analysis of Tyr-α1/2 revealed that this fraction exhibited a major peak at 2867 and several minor peaks at 2057, 2350, 2479, 2608, and 2737. All these peaks except at 2057 clearly originate from tyrosinated α1/2 and correspond to the modifications with one, two, three, and four glutamate units. The peak at 2057 corresponds to the Δ2 form of α1/2. The MALDI-TOF signals clearly indicate that the tetra-glutamylated form is the major species.

It should be mentioned that both isoforms of α1/2 exhibited several unidentified minor species in the mass spectograph. The peak at 2149 seems to be derived from the Δ2 form of α4 by the addition of a mass unit of 156, which is the mass of an arginine residue. On the other hand, the peaks at 2323 and 2387 seem to be derived from Δ2 forms of α4 and α1/2 by the addition of a mass unit of 200. Though it may be tempting to conclude that these modifications might be due to the addition of a glutamate (129 mass unit) and an alanine residue (71 mass unit), extensive mass spectrometric analyses are required to confirm this. It is not known whether the glycolating enzyme can use any other amino acid (such as alanine, serine, or histidine) as its substrate. Purification of this enzyme will be essential to shed more light on this.

It is interesting to note that the two forms of α1/2 differ remarkably in their assembly properties in the presence of MgCl2 (56). The tyrosinated α1/2 assembled quite normally, while the tyrosinated form did it poorly. Although it was predicted in my earlier work that these two forms might differ in their posttranslational modifications, the present MALDI-TOF studies clearly show that the major difference is in the glycolation/glutamylation status of the isoform. If one compares the major peaks for both the fractions, one can see that the tyrosinated form is mainly the biglycylated Δ2 form of α1/2, while the tyrosinated form is mainly the tetraglutamylated form of tyrosinated α1/2. It is conceivable that these two forms will differ significantly in the charge distribution at the C terminus. Since it is believed that the C termini of α- and β-tubulin is involved in the binding of MAPs to tubulin (59–61), polyglycation and polyglutamylation may represent modifications by which the assembly and disassembly of microtubules may be regulated in vivo.

These results are fairly consistent with the recent study by Vinh et al. (54), which shows that both dynamic cytoplasmic microtubules as well as stable axonal microtubules, can be glylated on each of the last four C-terminal glutamate residues of Glu437, Glu438, Glu439, and Glu441 in the β-tubulin sequence 427DATAEEGEFEEEGQ442. In both dynamic and stable microtubules, the majority of the β-tubulin contains six posttranslationally added glycine residues: two glycine residues on both Glu437 and Glu438 and one glycine residue on both Glu439 and Glu441.

In this context it should be mentioned that Redeker et al. (55) did not observe glycation in brain tubulin. They have studied the posttranslational modifications of α-tubulin from unfractionated PC-tubulin (tubulin purified by phosphocellulose chromatography) by purifying the C-terminal peptide on an arginine-Sepharose column. On the other hand, my affinity column separates the Δ2 form of α1/2 from its tyrosinated form. It is not known whether α4 also is glylated. Sequencing studies have identified α4 tubulin in a fraction (Fraction B) that contained both α1/2 and α4 as the tyrosinated forms. MALDI-TOF analysis of this fraction yielded many unknown species, which makes me believe that α4 may be subject to posttranslational modifications other than polyglycation and polyglutamylation. To get a clear picture on the posttranslational modification of α4, it must be separated from α1/2 prior to mass-spectrometric analysis.

At this point it is not clear about the number of glutamate residues modified by polyglycation. I have previously suggested that there might be more than one Glu residue that may be modified (56). The sequencing chromatogram identified residues Glu445, Glu447, and Glu449, which gave poor signals indicating that these residues are probably modified by polyglycation. Thus it seems likely that the residues Glu445, Glu447, and Glu449 are each modified by the addition of a glycol unit.

The tubulin molecule is subject to a large number of posttranslational modifications including phosphorylation, acetylation, tyrosination/detyrosination, deglutamylation, polyglutamylation and polyglycation (21). In general the functional significance of these modifications is not well understood. Tyrosination and detyrosination are thought to be involved, respectively, in the destabilization and stabilization of microtubules (57), and acetylation appears to stabilize microtubules also (58). It has been speculated that polyglutamylation of α and phosphorylation of the βII isotype of tubulin may regulate the interaction of microtubules with MAPs (59, 60). Recent
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studies have demonstrated that polyglycylation of tubulin modulated the interaction of tubulin with MAP1A, MAP1B, MAP2, and tau (61). Deamidation removes ß-tubulin from the tyrosination/detyrosination cycle and also leads to increased microtubule stability (43), while polyglycylation, hitherto found only in axonemal microtubules, may play a role in the formation and stabilization of those unusual microtubules (62). Since the MAPs-binding domain on tubulin is localized on the protofilaments H11 and H12 on the electron crystallographic structure of tubulin (63), it is possible that polyglycylation or polyglycylation can modulate the conformation of the binding domain drastically.

The results reported here have several implications for these issues. First they indicate that polyglycylation can occur in non-axonemal microtubules, thereby reopening the question of the function of this modification. As far as is known, in mammalian sperm only ß-tubulin is polyglycylated (49). Studies with Tetranychus ciliare tubulin suggest that, although both ß- and ß-tubulin are polyglycylated, only the polyglycylation of ß is important in axonemal function (61). Thus the finding reported here that mammalian brain ß/2 is polyglycylated is a novel observation and raises the possibility that polyglycylation of ß-tubulin serves a unique function. Second, these results show that tyrosinated and deamidated (Δ2) tubulins differ in more than just two residues, and that one form is polyglycylated and the other is polyglycylated. This is the first time that any posttranslational modifications of tubulin have been found to be correlated with each other. Third, they raise questions regarding the mechanism of the modifications. For example, is tubulin deamidated before being polyglycylated or vice versa? In either case the modifying enzymes must be specific for one particular form of tubulin. Does one modification make the other irreversible? The possibility now exists that there is a pathway of sequential modifications of the tubulin molecule. Fourth, these results suggest that some as yet unknown modifications may occur to the α4 isoform. Studies are in progress toward investigating these questions.

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