Posttranslational Modifications in the C-terminal Tail of Axonemal Tubulin from Sea Urchin Sperm*

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After proteolytic digestion of sperm tubulin from sea urchin Paracentrotus lividus, C-terminal peptides were isolated by chromatographic separations. The peptides were analyzed by Edman degradation and matrix-assisted laser desorption/ionization-time of flight mass spectrometry. About 70% of the isolated C-terminal peptides were unmodified. The remaining modified peptides have undergone a combination of numerous posttranslational modifications generating significant heterogeneity of sperm tubulin. α-Tubulin is modified by detyrosylation, release of the penultimate glutamate, polyglutamylation, and polyglycylation. Glycyl and glutamyl groups can coexist within one α-tubulin isoform. β-Tubulin undergoes polyglycylation but was not observed to be polyglutamylated. The number of units posttranslationally added reaches 11 and 32 glycy units on β- and α-tubulin, respectively. This is different from the polyglycylation of axonemal tubulin in Paramecium where up to 40 added glycy units were observed both on α- and β-tubulin.

The αβ tubulin dimer is the structural unit of microtubules that contribute to the maintenance of cell shape, chromosomal segregation during mitosis, axonemal transport, and cell motility. Both α- and β-tubulin subunits are encoded by multigene families (1, 2), the primary products of which can be extensively diversified by several posttranslational modifications. It has been reported that α-tubulin can be acetylated on Lys-40 (3), modified by detyrosylation/tyrosylation of the C terminus (4), with the possible excision of the penultimate glutamyl residue after detyrosylation (5). The class III β-tubulin isoform can be phosphorylated on Ser-444 (6, 7) and Tyr-437 (8). For both α- and β-tubulin two major polymodified peptides have been recently described: polyglutamylation (9) and polyglycylation (10). These two modifications consist of the addition of several amino acids to the γ-carboxyl group of a glutamyl residue located in the C-terminal part of the protein: one to at least six glutamates for polyglutamylation (9, 11–14), and 3–40 glycins for polyglycylation (Refs. 10 and 15). Polyglutamylation contributes widely to the high heterogeneity of mammalian brain tubulin. Polyglycylation was originally described on axonemal tubulin from Paramecium cilia (10). It was interesting to chemically characterize polyglycylation on axonemal tubulin from species other than Paramecium to ascertain the occurrence of this modification. Furthermore, since the glutamyl residues modified either by polyglutamylation or polyglycylation are close to each other, we were interested in determining whether these two modifications are mutually excluded within one tubulin molecule. Preliminary immunological studies, using GT-335 and AXO-49 monoclonal antibodies, have shown that tubulin from sea urchin Paracentrotus lividus sperm should be polyglutamylated (16) and polyglycylated (17). To answer these questions we characterized the different posttranslational modifications occurring in the C-terminal tail of sea urchin axonemal tubulin.

After its purification from sea urchin P. lividus spermatozoa, tubulin was digested either with endoprotease Asp-N or with thermolysin. The different proteolytic peptides were separated by HPLC chromatography and the acidic C-terminal peptides were characterized both by their amino acid sequence, using Edman degradation, and by their molecular mass, using MALDI-TOF mass spectrometry. It may be noted that the α-tubulin isoform isolated from spermatozoa axonemes of P. lividus and identified in this work has never been described before in this species. We determined the different modifications localized in the C-terminal tail of axonemal tubulin. We have observed that more than 60 and 80% of the isolated C-terminal peptides of α- and β-tubulin, respectively, are unmodified. Within the modified C-terminal peptides, the removal of the residue Tyr-451 is a predominant posttranslational modification of α-tubulin. Moreover, axonemal α- and β-tubulin can be glycylated; up to 12 glycy residues can be added. However, polyglutamylation was only observed on the C-terminal tail of α-tubulin, where up to six glutamyl residues can be added. It was also observed that α-tubulin can be both polyglutamylated and glycylated. From this work it appears that polyglutamylation and glycation are not mutually exclusive.

EXPERIMENTAL PROCEDURES

Chemicals—The following were purchased: Pipes and GTP (Boehringer Mannheim); Tris(hydroxyethyl)-aminomethane (Merck); EDTA, EGTA, dithiotreitol, and phenylmethylsulfonyl fluoride (Sigma); acetonitrile (Baker); and trifluoroacetic acid (Applied Biosystems).

Tubulin Purification—Purification of sea urchin P. lividus axonemal tubulin followed Gaenss et al. (18). Axonemes were a gift from Dr. P. Schmitter, unpublished results.

‡ The abbreviations used are: HPLC, high performance liquid chromatography; DEAE, diethylaminoethyl; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; Pipes, 1,4-piperazine diethanesulfonic acid.
Huitorel (Villefranche-sur-mer). They were stored in 10 mM Tris buffer, pH 7.4, 50% glycerol (w/v). Axonemes (10 mg/ml) were washed twice with washing buffer (10 mM Tris-HCl, pH 7.4, 1 mM dithiothreitol; 0.1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride) and pelleted at 15,000 × g for 10 min at 4 °C. The pellet was resuspended in a low ionic strength buffer (1 mM Tris-HCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) and dialyzed against 100 volumes of the same buffer overnight, using a dialysis membrane with a 3,500 M cut-off (Specta/Por). The sample was centrifuged at 80,000 × g for 30 min at 4 °C to pellet the microtubule doublets. The pellet was stored one night at −80 °C and resuspended in 10 mM Tris-HCl, pH 8.0, buffer. The sample was sonicated and centrifuged at 180,000 × g for 10 min at 4 °C. The supernatant, containing soluble axonemal tubulin, was collected.

Proteolytic Digestion—Proteolysis of purified tubulin (1 mg) by endoproteinase Asp-N (Boehringer Mannheim) was carried out in 50 mM Tris-HCl, pH 8.0, buffer with a 1/400 enzyme/substrate ratio (w/w) for 6 h at 36 °C. Digestion was stopped by placing the sample at −80 °C.

Proteolysis of purified tubulin (1 mg) by thermolysin (Boehringer-Mannheim) was carried out in thermolysin buffer (50 mM Tris-HCl, pH 8.0, containing 0.1 mM CaCl₂) with a 1/20 enzyme/substrate ratio (w/w) for 5 h at 36 °C. Digestion was stopped by placing the sample at −80 °C.

HPLC Separation—Before separation on the HPLC column, the sample was centrifuged at 12,000 × g for 2 min, and the supernatant was injected. Purification of the carboxyl-terminal acidic peptides of tubulin was performed by anion exchange chromatography followed by reverse phase chromatography. The elution was monitored at 214 nm. The DEAE separation conditions have been described previously (14). The reverse phase HPLC apparatus was composed of a 140B solvent delivery system (Applied Biosystems) and a Waters 990 (Millipore) detector monitored with M 990 photodiode array detector software. The reverse phase column (Aquapore RP-300; 7 μm, 220 × 2.1 mm) was eluted at room temperature at a flow rate of 200 μl/min. Solvent A was deionized water, 0.1% trifluoroacetic acid, and solvent B was 80% acetonitrile in water, 0.09% trifluoroacetic acid (v/v). The gradient used was 1% B for 10 min, then from 1 to 5% B for 1 min followed by a linear gradient between 5 and 60% B for 55 min.

Amino Acid Sequencing—Peptides were sequenced by automated Edman degradation using a 470A gas-liquid sequenator (Applied Biosystems).

Mass Spectrometry—Samples were prepared by mixing 0.5 μl of matrix (50 mM solution of α-cyano-4-hydroxy-cinnamic acid in 1/1 ethanol/acetonitrile) with 0.5 μl of peptide (1–4 pmol in 20% acetonitrile, 0.1% trifluoroacetic acid). The mixture was loaded onto a stainless steel target and allowed to dry at room temperature. The external standards used for calibration were bradykinin and bovine insulin. The mass spectrometer was a Fisons Instruments VG Analytical time-of-flight instrument (model Tofspec) equipped with a 337-nm laser source and a magnetic sector mass spectrometer. Analysis of the most illustrative DEAE fractions is presented in this work.

RESULTS

Isolation of the C-terminal Peptides from Axonemal Tubulin—After its purification from sea urchin P. lividus spermatozoa, axonemal tubulin was digested by two proteolytic enzymes, endoproteinase Asp-N, or thermolysin (see “Experimental Procedures”). Peptides were separated on a DEAE column (Fig. 1, panels A and B, respectively). As was previously observed (10, 12, 14) the C-terminal peptides, characterized by their high acidity, were eluted during the second part of the linear gradient, in the fractions designated DEAE-1 to -14. These fractions were separately collected and further purified on a reverse phase column. The isolated peptides were characterized both by their amino acid sequence by Edman degradation and by their molecular mass by MALDI-TOF mass spectrometry. Analysis of the most illustrative DEAE fractions is presented in this work.

Fig. 2A shows the reverse phase HPLC analysis of the major acidic DEAE fraction, designated DEAE-5. This chromatographic pattern, with the elution of two major peaks, is also found for the reverse phase separations of fractions DEAE-1 to -7. The sequence of the peptide eluted under peak 1 of the reverse phase separation matches with the C-terminal peptide of β-tubulin beginning at position 427: 427DATAEEGFDEEEGDEEA 457. The mass spectrum (Fig. 2B) of this peptide reveals a molecular ion with a mass-to-charge ratio m/z = 2324.1, which corresponds to the unmodified β-tubulin peptide (427–447) with one Na⁺ adduct (MNa⁺ calculated average mass = 2324.1). The differences between the calculated average mass and the experimental mass determination range from 0.1 to 1.7 Da, consistent with the accuracy of MALDI-TOF mass spectrometry in reflect on mode. Sequence of the peptide eluted under peak 2 was identified as the C-terminal peptide of α-tubulin beginning at position 424: 424DLAA[EK-DYEEVGDSVGEAEEGEEY 451. The mass spectrum (Fig. 2, panel C) of this peptide shows a molecular ion at m/z = 3105.8, which corresponds to the unmodified α-tubulin C-terminal peptide (424–451) (MH⁺ calculated average mass = 3105.2).

Unbranched Posttranslational Modifications Identified on C-terminal Peptides of α-Tubulin—Within the posttranslational modifications affecting the C-terminal tail of α-tubulin, the unbranched modifications (i.e. deamidation of asparagine and removal of the penultimate glutamyl residue (5)) are here distinguished from the polymodifications (i.e. polyglycylamyla 9) and polyglycylation (10)).

Two α-tubulin C-terminal peptides isolated by reverse phase separation and analyzed both by amino acid sequencing and mass spectrometry, beginning at amino acid Asp-424, were found to correspond to unbranched modified peptides. The major peptide, purified from fraction DEAE-3 gave a molecular ion at m/z = 2941.4 (MH⁺ calculated average mass = 2942.0)
corresponding to the detyrosylated C-terminal peptide of \(\alpha\)-tubulin (424–450). The minor peptide, purified from fraction DEAE-2, gave a molecular ion at \(m/z = 2812.4\) (MH\(^+\) calculated average mass = 2812.9) corresponding to the C-terminal peptide of \(\alpha\)-tubulin without the penultimate glutamyl residue Glu-449 (424–449) (data not shown).

Posttranslational Polymodifications Identified on C-terminal Peptides of \(\alpha\)-Tubulin—Sequences of the \(\alpha\)-tubulin peptides, purified on reverse phase column from fraction DEAE-7, were \(^{424}\)DLAALEKDYEEVGVDSEVEGEA\(^{444}\). No aminocyl residue was detected after Ala-444. According to previous results, the undetected glutamyl residue corresponding to position 445 could be posttranslationally modified by glutamylation (9) or glycylation (10). Fig. 3A presents a mass spectrum of these peptides. Two series of molecular ions can be observed. The first one begins with a molecular ion at \(m/z = 3106.5\), which corresponds to the C-terminal peptide of tyrosylated \(\alpha\)-tubulin (424–451) (MH\(^+\) calculated average mass = 3105.2). The three other peaks of this series are separated from each other by increments of 129 atomic mass units, which corresponds to one glutamylation. So the molecular ions observed in this first series correspond to the C-terminal peptides of tyrosylated \(\alpha\)-tubulin with 0–3 glutamyl units added posttranslationally.

The second series begins with a molecular ion at \(m/z = 3589.2\), corresponding to the detyrosylated \(\alpha\)-tubulin peptides (424–450) with 5 glutamyl units posttranslationally added (MH\(^+\) calculated average mass = 3587.5). The following ion represents an increment of 129 atomic mass units. This second series corresponds to C-terminal peptides of detyrosylated \(\alpha\)-tubulin bearing 5–6 glutamyl units posttranslationally added. Thus, analysis of these peptides shows that the C-terminal tail of \(\alpha\)-tubulin can be glutamylated and that the glutamyl residues are posttranslationally added at least on the \(\gamma\)-carboxylic moiety of Glu-445. Among all the isolated peptides, it was observed that tyrosylated peptides can bear 1–6 glutamyl units and that detyrosylated peptides can have 1–3 additional glutamyl units.

Sequences of the \(\alpha\)-tubulin peptides, purified on reverse phase column from fraction DEAE-2, were \(^{424}\)DLAALEKDYEEVGVDSEVEGEA\(^{444}\). As previously, the undetected glutamyl residue corresponding to position 445 could be posttranslationally modified. Fig. 3B presents the mass spectrum of these peptides. Again, two series of molecular ions can be observed. In the major series, six molecular ions are each separated by 57 atomic mass units, which is the mass of one glutamyl residue. For each series, the \(m/z\) value of the first ion is presented. Adducts of Na\(^+\) (●) and K\(^+\) (●) are observed. \(\alpha\), C-terminal peptide of tyrosylated \(\alpha\)-tubulin (424–451), \(\alpha\)Y, C-terminal peptide of tyrosylated \(\alpha\)-tubulin (424–450), and \(\alpha\)Y/F, C-terminal peptide of tyrosylated \(\alpha\)-tubulin (424–451) eluted under peak 1 and 2, respectively.
C-terminal peptides of \( \alpha \)-tubulin corresponding to position 445 could be posttranslationally modified by their amino acid sequence and mass. Mass spectrometry of C-terminal peptide \( \alpha \)-tubulin (424–450) is presented. Three molecular ion series, corresponding to glycylated peptides, are observed. For each series, the m/z value of the first ion is presented. Adducts of Na\(^{+}\) (●) and K\(^{+}\) (○) are observed.

glycyl residue. This mass increment is typical of polyglycylated peptides (10). The first molecular ion of this series has an m/z = 3113.8, which corresponds to the C-terminal peptide of \( \alpha \)-tubulin (424–450) with the addition of 3 glycyl units (MH\(^{+}\) calculated average mass = 3113.1). The following ions correspond to the same peptide bearing 4–8 glycyl units. A minor series of five molecular ions, also presenting increments of 57 atomic mass units, can be observed. The first ion at m/z = 3527.9 corresponds to the detyrosylated \( \alpha \)-tubulin peptide (424–450) with the addition of one glutamyl unit and 8 glycyl units posttranslationally added (MH\(^{+}\) calculated average mass = 3527.5). In this series the level of polyglycylation reaches 12 glycyl units. Hence, analysis of these peptides shows that the C-terminal tail of \( \alpha \)-tubulin can be glycylated and that the glycyl units are posttranslationally added at least on the \( \gamma \)-carboxylic function of the Glu-445. It was observed that \( \alpha \)-tubulin bearing 2–8 glycyl units is always detyrosylated.

Sequences of the \( \alpha \)-tubulin peptides, purified on reverse phase column from fraction DEAE-3, were DEALEKDYEEVGDVGSVGEA\(^{434}\). Again, the undetected glutamyl residue corresponding to position 445 could be posttranslationally modified. Fig. 4 shows the mass spectrum of these C-terminal peptides of \( \alpha \)-tubulin. In this extremely complex mass spectrum three series of molecular ions are observed. In the major series, six molecular ions are each separated by 57 atomic mass units. The first molecular ion of this series has a ratio m/z = 3299.4, which corresponds to the C-terminal peptide of detyrosylated \( \alpha \)-tubulin (424–450), with the addition of one glutamyl unit and 4 glycyl units (MH\(^{+}\) calculated average mass = 3299.3). The following molecular ions correspond to the addition of 5–9 glycyl units. The two minor series correspond also to the C-terminal peptide of detyrosylated \( \alpha \)-tubulin. The first one, beginning with the molecular ion at m/z = 3056.0, corresponds to the addition of 2–4 glycyl units (MH\(^{+}\) calculated average mass = 3056.1) without the addition of a glutamyl unit. The second series, beginning with the molecular ion at m/z = 3656.9, corresponds to the addition of two glutamyl and 8–11 glycyl units (MH\(^{+}\) calculated average mass = 3656.6). When polyglutamylation and polyglycylation were detected together, it was observed that the addition of one glutamyl unit occurs with the addition of 2–12 glycyl units and that addition of 2 glutamyl units occurs with the addition of 8–10 glycyl units.

Characterization of peptides obtained by proteolysis of axonemal tubulin with endoproteinase Asp-N reveals that the C-terminal tail of \( \alpha \)-tubulin can be both polyglutamylated and polyglycylated and that these two posttranslational modifications can occur separately or together on one molecular species.

Posttranslational Modifications Identified in C-terminal Peptide of \( \beta \)-Tubulin—Proteolysis of axonemal tubulin with endoproteinase Asp-N and analysis of peptides eluted in the last part of the DEAE separation led only to the characterization of unmodified C-terminal peptides of \( \beta \)-tubulin. To specify the posttranslational modifications of the C-terminal tail of the \( \beta \)-subunit, proteolysis of tubulin was performed with thermolysin. Peptides were purified and characterized as described previously (14). Fig. 5A shows the analysis of fraction DEAE-13 by reverse phase. Peaks eluted are designated TL-1 to -3. The sequence obtained for the peptide eluted under peak TL-1 was DEALEKDYEEVGDVGSVGEA\(^{434}\), matching with a C-terminal sequence of \( \beta \)-tubulin beginning at position 430. Since no aminoacyl residue can be detected after Asp-437, this peptide could be posttranslationally modified at least on Glu-438. A mass spectrum of this peptide is presented in Fig. 5B. A series of three molecular ions can be observed, each separated by increments of 57 atomic mass units. Molecular ion at m/z = 2122.5 corresponds
to the C-terminal β-tubulin peptide (430–445) with the addition of 4 units of 57 atomic mass units, and one adduct of Na\(^+\) (MNa\(^+\) to the C-terminal tail of the β-tubulin peptide (430–445) bearing 4–6 glycyl units posttranslationally added. These glycyl residues should be linked at least at Glu-438 as a site of modification. Sequences of the peptides eluted under peak TL-2 and -3 match with the C terminus of β-tubulin (430–444) and (430–447), respectively. Mass spectrometry analysis of these peptides revealed that they are unmodified (results not shown).

By analysis of all the acidic peptides obtained after proteolysis of tubulin with thermolysin it appears that the C-terminal β-tubulin peptides can be polyglycylated with the addition of 1–11 glycyl units at Glu-438. No other posttranslational modification was detected on the C-terminal tail of the β-subunit.

**DISCUSSION**

In this study, posttranslational modifications affecting the C-terminal tail of both α- and β-tubulin purified from P. lividus spermatozoa axonemes are analyzed and characterized. It must be noted that the α-tubulin isoform identified in this work has never been described before in this species (see Table I). The relative amount (%) of each C-terminal peptide was calculated by dividing the amount of peptide in each reverse phase fraction (estimated by the area of the peak detected at 214 nm) by the sum of C-terminal peptides of α- or β-tubulin isolated. It is shown that the majority of isolated C-terminal peptides of both α- and β-tubulin are unmodified (more than 60 and 80% respectively; see Table II).

The C-terminal tail of α-tubulin can be polyglutamylated with the addition of 1–6 glutamyl residues and polyglycylated with the addition of 1–8 glycyl residues. Glutamyl and glycyl residues appear to be added at least on the γ-carboxyl function of Glu-445. Primary sequence cannot be determined beyond this position, and thus the possibility of the existence of other sites of modifications cannot be excluded. A minor fraction of C-terminal peptides of α-tubulin appears to be both glutamylated and glycylated. For these peptides at least Glu-445 appears to be a site of modification, either for glutamylation or glycylation. Moreover the possibility cannot be ruled out that glutamyl and glycyl residues can be added at the same site, i.e., Glu-445, and thus within the same lateral chain. These results show that these two posttranslational polymodifications can coexist within one α-tubulin molecule. Approximately 19% of the isolated C-terminal peptides of α-tubulin are glutamylated, 4% are glycylated, and another 4% are both glutamylated and glycylated (Table II).

Within the unbranched posttranslational modifications, detyrosylation was the most abundant detected, approximately 30% of C-terminal peptides from α-tubulin being detyrosylated. It was observed that detyrosylated and tyrosylated α-tubulin are polyglutamylated almost in the same proportion (see Table II). This is in agreement with previous results showing that polyglutamylation and detyrosylation/tyrosylation are two posttranslational modifications occurring independently on α-tubulin (19). However, the number of added glutamyl residues detected on detyrosylated α-tubulin is twice that of the tyrosylated form (6 versus 3). Furthermore, polyglycylated α-tubulin with or without glutamylation was always detyrosylated. A very small fraction of C-terminal peptides of α-tubulin were deglutamylated by release of the Glu-450 (L2-tubulin).

For C-terminal peptides of β-tubulin the only detected posttranslational modification was polyglycylation: 1–11 glycyl residues can be added. At least the γ-carboxyl function of Glu-438 should be one site of modification. As previously, the existence of other sites of glycylation cannot be ruled out. Less than 20% of the C-terminal peptides of β-tubulin are glycylated (Table II).

Now it is important to determine if the sites of modification described in this work are the only sites, particularly in the case of α-tubulin where the two polymodifications can occur simultaneously on the same molecule.

The axonemal tubulin from sea urchin spermatozoa appears to be a structure where glutamylation and glycylolation coexist and moreover can modify the same α-tubulin molecule. The possible combination of polyglutamylation with polyglycylation leads to a dramatical increase in the molecular complexity of the system. However, in the present work it appears that more than 60% of axonemal tubulin of sea urchin spermatozoa is unmodified. Thus, if each modification has a specific function,
only a few modified isoforms within a microtubular structure, such as the axoneme, may confer specific functions to individual microtubules.

In addition to the nature of the posttranslational modifications affecting the C-terminal tail of α- and β-tubulin and their relative proportion, it is also important to specify their distribution along the axonemal structure. Immunofluorescence studies with specific antibodies directed against posttranslational epitopes have been carried out [17, 20, 21]. Furthermore, the effect of anti-modification antibodies was tested on different motility parameters in reactivated sea urchin spermatozoa. 

The two polymodifications, glutamylation and glycylation, were originally described in two very distinct systems: mammalian brain, where all expressed tubulin isoforms can be polyglutamylated, and the axoneme of Paramecium cilia where all tubulin isoforms are polyglycylated. Recently Rüdiger et al. [15] have shown that in axonemal tubulin from bull sperm, polyglutamylation was described on β-tubulin, while polyglycylamylation appeared to be confined to α-tubulin. From the present study carried out with axonemal tubulin from sea urchin it appears that these two polymodifications can coexist on one α-tubulin molecule.

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