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Class II tubulin, the major brain β tubulin isotype is polyglutamylated on glutamic acid residue 435

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Protein sequencing shows that porcine brain tubulin retains the N-terminal sequences of α and β tubulin after a mild treatment with subtilisin. C-terminal peptides released by subtilisin were purified and characterized by automated Edman degradation and mass spectrometry. We confirm the polyglutamylation of α tubulin on glutamic acid residue 445 reported by others and show in addition that class II β tubulin, the major β tubulin isotype of adult brain, is also polyglutamylated. The substitution is restricted to glutamic acid residue 435. Thus all major tubulin isotypes of adult brain are subjected to polyglutamylation.

Glutamylation; Mass spectrometry; Posttranslational modification; Subtilisin; Tubulin

1. INTRODUCTION

The αβ tubulin heterodimer is the major component of microtubules. In higher vertebrates α and β tubulins are encoded by a multigene family which has been characterized in detail by cDNA cloning. Both α and β tubulins contain an acidic domain at the C-terminal end which is dominated by glutamic acid (for review see [1]). Mild treatment of brain tubulin with subtilisin truncates both α and β chains. It removes the acidic stretches and is thought to produce a C-terminal 4 kDa fragment from both chains [2,3]. Although these fragments have attracted great interest as binding sites for MAP2, τ proteins and brain cytoplasmic dynein [2–6] they have not been directly characterized by amino acid sequencing. In addition, it is not known by sequencing whether the subtilisin treatment leaves the N-terminal ends of the tubulins fully intact. In spite of this shortcoming, various results prior to MAP2 and τ binding sites in the C-terminal regions. There is however some disagreement as to the precise location of these sites along both polypeptide chains. They either involve the acidic regions of β tubulin [5], of both α and β tubulin [6], or the sequences preceding the acidic regions of both α and β tubulin [7,8]. During a not yet completed analysis of the subtilisin treatment of brain tubulin, we isolated several peptides covering the C-terminal regions of α and β tubulins. Sequence analysis and mass spectrometry confirm the recent reports that α tubulin is polyglutamylated at glutamic acid residue 445 [9–12] and show that this modification is also present on glutamic acid residue 435 of class II β tubulin, the major β tubulin isotype in adult brain.

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2. MATERIALS AND METHODS

Purification of microtubule protein [13] and tubulin tyrosine ligase [14] from porcine brain followed published procedures. Tubulin free of associated proteins was obtained by phosphocellulose chromatography [15]. Up to 8 mg of tubulin were labelled to saturation using tubulin tyrosine ligase and [^{14}C]tyrosine (Amersham; specific radioactivity 30 mCi/mmol). Labelling conditions have been described [14]. Excess radioactivity was removed by gel filtration through a PD10 column (Pharmacia).

Tubulin (1–2 mg/ml in 0.1 MES, 1 mM EGTA, 1 mM MgCl, 1 mM GTP adjusted to pH 6.8 with KOH) was treated at 30°C with subtilisin/Carlsberg (Sigma) at 1% by weight using the conditions described by Sacket et al. [16]. Aliquots removed after 0 to 40 min were made 0.2 mM in PMSF and analyzed by SDS-PAGE. To improve the separation of α and β tubulin, SDS preparations containing 30% of myristyl- and cetylsulfate (Sigma) were used and the buffers for the stacking and the separation gels were made 6 M in urea. Separated polypeptides were either stained with Coomassie brilliant blue or transferred electrophoretically onto polyvinylidenedifluoride (PVDF) membranes (Problot; Applied Biosystems) and sequenced using an Applied Biosystems gas phase sequenator (model A470) or a Knauer sequenator (model 810). Both instruments were equipped with an on line PTH-amino acid analyser.

Digests were stopped with PMSF and then kept for 30 min on ice to depolymerize microtubules. They were centrifuged and then applied at 4°C on a Sephadex G50 column (1.5 × 34 cm) equilibrated with 20 mM ammoniumacetate. Fractions were monitored by optical density at 220 nm and by liquid scintillation counting. Fractions containing the radioactive peptides were lyophilized and subjected to two dimensional fingerprinting on Whatman 1 MM paper. Separation was by pH 6.5 electrophoresis (10% pyridine, 0.5% acetic acid) and descending chromatography (*n*-butanol/water/pyridine/acetic acid 15:10:12:3). Peptides visualized by 0.005% fluorescamine in acetone or by autoradiography were eluted with 20% acetonitrile containing 0.02% TFA. An additional fingerprint yielded peptide 2 after a light treatment with

ninhydrine. Samples were dried, dissolved in solvent A (0.1% TFA in water) and subjected to HPLC using a Vydac C18 Tp 52 reversed phase column. After injection of the sample the column was first washed with solvent A. Peptides were eluted with a 30 ml linear gradient of 1 to 50% solvent B (50% acetonitrile, 0.1% TFA in water) at a flow rate of 1 ml/min. The elution profiles were monitored by optical density at 214 nm and radioactive peptides were detected by liquid scintillation counting. Peptides were sequenced as described above.

The acidic C-terminal peptides were methylated at their carboxylate groups at room temperature by acetylchloride in methanol as described [17]. Mass spectrometry of the esterified peptides was performed by fast atom bombardment mass spectrometry. Fast atom bombardment (FAB) mass spectra were obtained on a Kratos MS 50 RF TC high field magnet instrument equipped with a Xenon atom gun (Ion Tech, 8 keV). The samples were dissolved in acetic acid (0.1%) and thioglycerol (Sigma) was used as matrix on a steel tip. The spectra were recorded at an accelerating potential of 8 kV with a magnet scan rate of 30 s/dec at a resolution of 2500.

3. RESULTS

Porcine brain α and β tubulin separated by SDS-PAGE (Fig. 1, slot 1) were transferred on a PVDF membrane and subjected to sequence analysis. The N-terminal sequence of α tubulin — MREXISIHVGRQ-AGVQI — agrees with the earlier protein data [18] since the unmodified cysteine present at position 4 cannot be identified under standard conditions. The corresponding sequence of β tubulin — MREIVH(I/L)QAGQXG-NQ — showed the presence of at least 2 isotypes. At position 7 isoleucine and leucine were found at 70 and 30% respectively. The first sequence fits the previous protein data [19] and corresponds in the catalogue of β isotypes deduced from cDNA cloning [1] to classes II and III, which can only be distinguished in other regions of the molecules. The second sequence fits the class IVa isotype [1]. Again as in the α chain the unmodified cysteine (X) was not identified.

Treatment of porcine brain tubulin with subtilisin

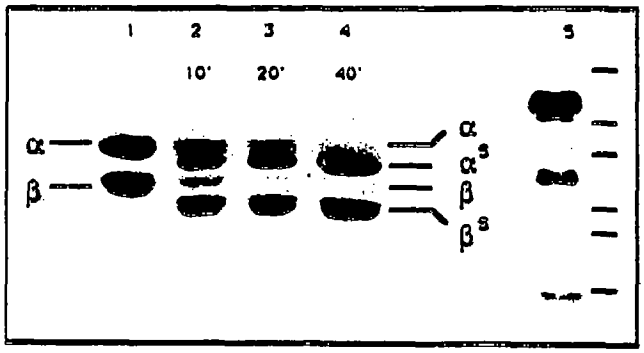

Fig. 1. SDS-PAGE of brain tubulin and the time course of subtilisin treatment. Purified tubulin (slot 1) was treated with subtilisin for 10 to 40 min (slots 2 to 4). α and β polypeptides from slot 1 and the polypeptides of slot 4 were blotted on to PVDF and subjected to sequence analysis (see section 3). Slot 5 shows a more extensively degraded preparation which was run on a different occasion (see text). Here the normal 15% gel system without the additions described in section 2 was used. Therefore the truncated α and β chains were not resolved.

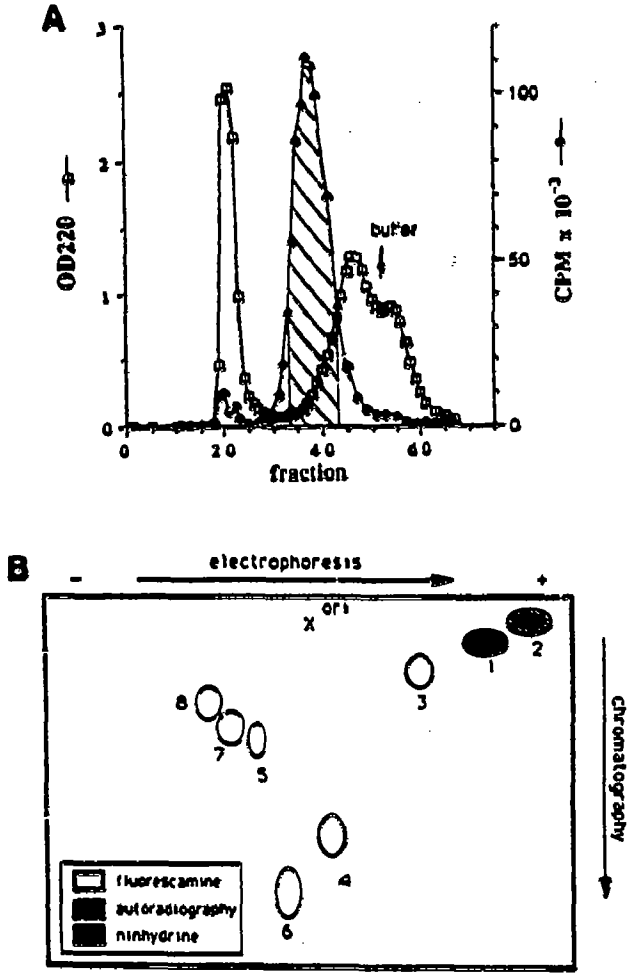

Fig. 2. Isolation of tubulin peptides released by subtilisin. Tubulin previously labelled by tubulin tyrosine ligase with [^{14}C]tyrosine was treated with subtilisin and the digest was stopped by PMSF. Panel A shows the separation profile on Sephadex G 50 monitored by optical density and liquid scintillation counting. The peak of small peptides is marked. Corresponding fractions were pooled and subjected to two-dimensional fingerprinting (panel B). The origin of application is marked (ori). Separation was by pH 6.5 electrophoresis (horizontal arrow) and by descending chromatography. Peptide detection is indicated by the symbols given in the insert.

leads in a time dependent manner to a conversion of α and β into α^s and β^s polypeptides which have reduced apparent molecular weights when analyzed by SDS-PAGE (Fig. 1). Sequence analysis showed that the truncated α^s and β^s tubulin chains retain the N-terminal sequences of α and β tubulin respectively. Thus subtilisin induced truncation removes in the first stages of digestion only C-terminal sequences. At later stages of digestion or under more pronounced proteolytic conditions, additional bands in the apparent molecular mass range of 14 to 30,000 became more pronounced (Fig. 1, slot 5; see also [16]).

To analyze the C-terminal cleavage sites, we used a preparation of tubulin previously tyrosinated with [^{14}C]tyrosine by tubulin tyrosine ligase, since this treat-

ment introduces a C-terminal tyrosine into some of the α chains [12,20–23]. After subtilisin treatment for 35 min the digest was stopped with PMSF and passed through Sephadex G50. Fractions containing [^{14}C]tyrosine were pooled (Fig. 2A) and subjected to two-dimensional fingerprinting (Fig. 2B). Peptides recovered were further purified by HPLC and characterized by sequence analysis (Fig. 3). In addition to C-terminal peptides arising from α and β tubulins (peptides 1a, 1b and 2) and the preceding α-peptide (residues 429 to 438; peptide 3) we isolated several peptides (Fig. 3) which cover more interior positions of the α and β chains. They arose due to overdigestion with subtilisin since a corresponding gel analysis of the original digest showed not only the α* and β* bands but also appreciable amounts of polypeptides in the molecular mass range of 14 to 30,000 (Fig. 1, slot 5).

Peptides 1a and 1b cover the C-terminal sequences of the non-tyrosinatable and the tyrosinated α tubulin forms, respectively [10]. The glutamic acid residue 445 predicted by cDNA sequences for peptide 1b could not be identified by the peptide sequencing (Fig. 3). Instead of the expected PTH-glutamic acid no PTH derivative was detected at this step but the sequence continued as expected past this step. Mass spectrometry documented the oligo-glutamyl substitution of glutamic acid residue 445 recently described in detail by Eddé et al. [9]. Up to three additional glutamyl residues were identified in peptide 1b (Fig. 4, middle panel). For peptide 1a, which lacks the C-terminal tyrosine and some of the preceding glutamic acid residue, we followed the interpretation of Paturle-Lafanechère et al. [10]. If the glutamic acid residue 449 is absent, up to three glutamyl-substitutions of residue 445 are indicated by mass spectrometry (Fig. 4, upper panel). Alternatively in peptides containing the glutamic acid residue 449, the maximal number of glutamyl substitutions is reduced to 2. More important are the results on peptide 2. Its sequence is in perfect agreement with the C-terminal sequence of the class II β tubulin isotype predicted by rat and mouse cDNA clones [24,25]. Position 2 of the peptide, corresponding to glutamic acid residue 435 in the predicted sequence, yielded again no PTH-amino acid. Mass spectrometry showed glutamylation by two, three and four glutamyl residues respectively (Fig. 4, lower panel). An additional very minor peak has a molecular weight consistent with 5 glutamyl residues attached to glutamic acid residue 435 (data not shown).

peptide	sequence	position in chain
1 a	SVEGEGXEEG (E)	α 439 -(449)
1 b	SVEGEGXEEGEEY	α 439 - 451
2	GXFEEEEGEDE	β 434 - 435
3	EKDYEEVGVD	α 429 - 438
4	YQPPTVVPGGDLA	α 357 - 369
5	KIREEYPDR	β 154 - 162
6	KLTTPTYGDL	β 216 - 225
8	RRNLDIERPTYTN	α 214 - 226

Fig. 3. Amino acid sequences of the isolated tubulin peptides. Peptides recovered from the fingerprint (Fig. 2B) were subjected to HPLC purification. Sequences were obtained by automated Edman degradation. X marks positions at which no PTH-amino acid was detected (see text). The location of each peptide along the polypeptide chains is given at the right. Peptide 7 was homologous to peptide 8 and not completely sequenced.

4. DISCUSSION

In the course of an analysis on the C-terminal truncation of porcine brain tubulin by subtilisin we found that class II β tubulin, the major β tubulin isotype in adult brain, is glutamylated on glutamic acid residue 435 (Fig. 4). This type of posttranslational modification was first reported two years ago for neuronal α tubulin. Here it involves the successive addition of glutamyl residues starting with the γ-carboxyl group of glutamic acid 445 [9]. Subsequent work confirmed that up to six glutamyl residues, yielding a peptidic side chain off the main polypeptide chain, can be monitored by mass spectrometry [11]. Glutamylation of α-tubulin and its C-terminal tyrosination are independent events [10,12] and the α tubulin variant, which cannot be tyrosinated, seems to lack glutamic acid residue 449 of the main α tubulin polypeptide chain [10]. Glutamylation is not restricted to neuronal α tubulin. A recent report shows that class III β tubulin, a β tubulin isotype found in moderate amounts in adult brain, is glutamylated at glutamic acid 438 and up to six glutamic acid residues can be introduced as side chain [26]. We have now extended this observation to class II β tubulin, the major β tubulin isotype of adult brain. Although we have found up to four glutamyl side chain residues on glutamic acid 435, a very minor peak of the mass spectrum is compatible with a five residue substitution. In addition, we note that potential C-terminal peptides carrying a higher degree of glutamyl substitution may have escaped our isolation procedure.

The combined results show that all major tubulin isotypes of adult brain are extensively glutamylated and that this progressive polyglutamylation explains major aspects of the pronounced tubulin heterogeneity observed by two dimensional gel analysis [9,11,27,28].

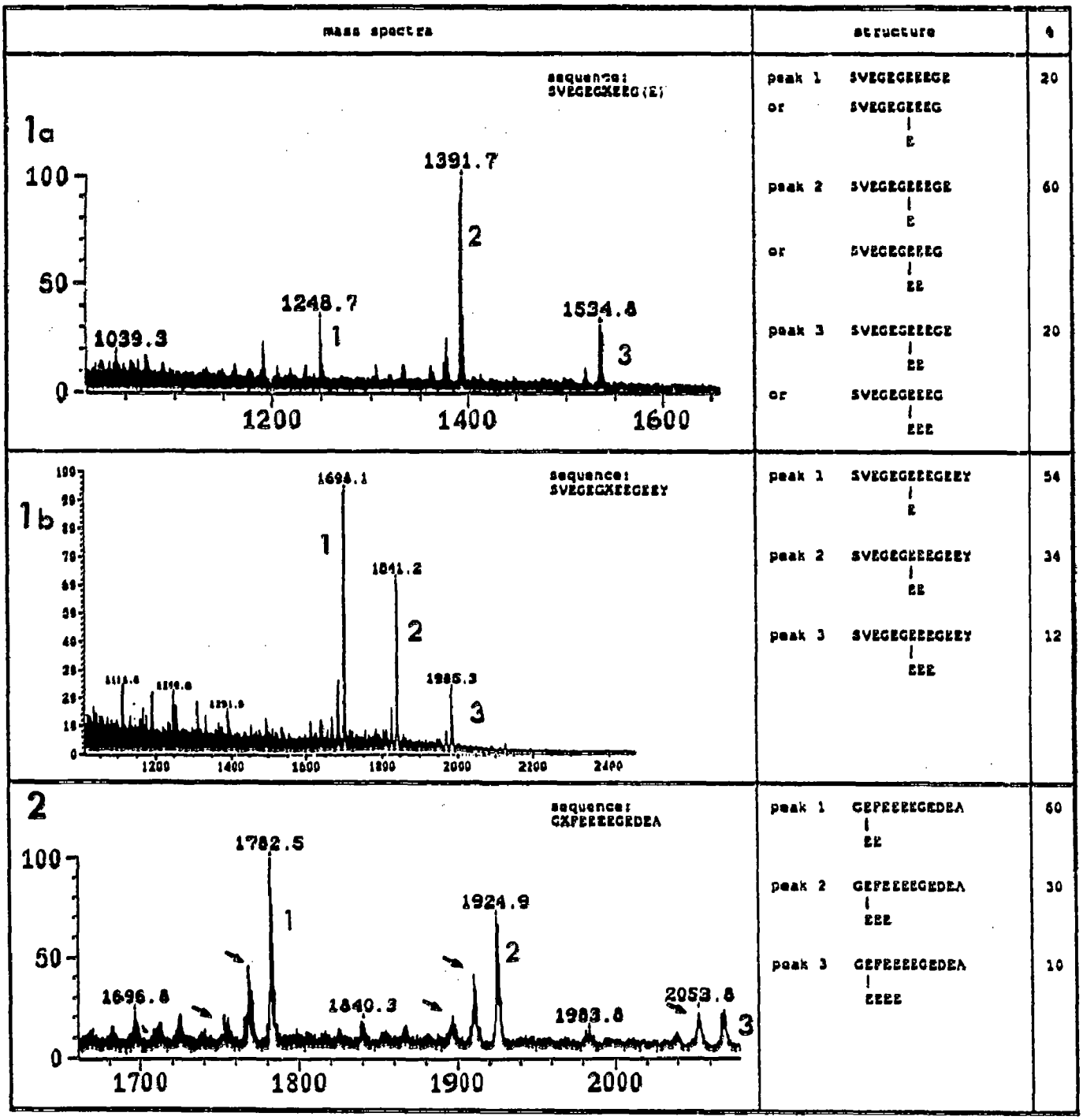

Fig. 4. Mass spectra of the carboxymethylated peptides 1a, 1b and 2. Mass spectra of the three peptides were recorded by FAB mass spectrometry. Masses of the protonated molecular ions $(M+H)^+$ are indicated. Arrowheads point to positions of incompletely methylated peptides (indicated only in the third panel). Mass-increments of 143 due to additional methylated glutamyl groups are found in all three spectra. Mass ranges are indicated. Structures deduced from the peptide sequencing and the mass spectra are given. The relative abundance of each species was calculated directly from peak height.

While the enzyme(s) responsible for tubulin glutamylation are still unknown, current results on three C-terminal tubulin peptides show that the modification is restricted in each case to a single glutamic acid residue of the polypeptide chain. When this residue is encountered in amino acid sequence analysis, no PTH-residue is identified in the standard chromatogram. In the three sequences established, SVEGEGEEEGEEY(α), GEFEEEEGDEA(β II) and YEDDEEESEAQGPK(β III) the substitution occurs in a sequence stretch already rich

in glutamic acid but involves only the single glutamic acid residue, which is underlined. While the functional importance of polyglutamylation of brain tubulin is not known, the acidic C-terminal domains of α and β tubulin are thought to be involved in binding of microtubule-associated proteins [2,5–8] and possibly in the Ca^{2+} sensitivity of microtubules [7,29].

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