Characterization of a Major Brain Tubulin Variant Which Cannot Be Tyrosinated†

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ABSTRACT: Brain tubulin preparations contain an abundant type of tubulin which does not undergo the normal cycle of tyrosination–detyrosination, and whose nature is still unknown. We have used peptide sequence analysis and mass spectrometry combined with immunological procedures to show that this non-tyrosinatable tubulin has a specific primary structure. It differs from the tyrosinated isotype in that it lacks a carboxy-terminal glutamyl-tyrosine group on its α-subunit. Thus, non-tyrosinatable tubulin originates from a well-defined posttranslational modification of the tubulin primary structure which is located at the expected site of activity of tubulin tyrosine ligase. This probably accounts for the reason why it cannot be tyrosinated. The significance of this abundant brain isotubulin and the metabolic pathway involved in its formation remain to be elucidated. This should shed light on the relation between the structural diversity of the carboxy terminus of α-tubulin and the regulation of functional properties of microtubules.

Tubulin has a high abundance in the brain, representing about 20% of the total proteins (Schmitt et al., 1977; Hiller & Weber, 1978), and this reflects the importance of microtubules in neuronal functions [for reviews see Dustin (1984), Bartlett Bunge (1986), and Vallee and Shpetner (1990)]. A specific property of tubulin, which is not shown by other proteins, is a reversible posttranslational modification of its primary structure. The carboxy-terminal amino acid of α-tubulin, tyrosine, can be cyclically removed and readded to the protein. This results in tubulin which is either tyrosinated (Tyr tubulin) or detyrosinated (Glu tubulin). Another type of tubulin which is non-tyrosinatable (N.Tyr tubulin) is also known to occur. The latter is the most abundant type and accounts for about half of the protein in adult brain tubulin preparations (Barra et al., 1980). However, its nature is still unknown, and this poses questions as to its function and structure in the brain.

The tyrosination–detyrosination cycle involves the action of a tubulin carboxypeptidase which removes the carboxy-terminal tyrosine from α-tubulin (Hallak et al., 1977; Argaraña et al., 1978, 1980) and a tubulin tyrosine ligase which subsequently readds a tyrosyl group, in an ATP-dependent reaction (Raybin & Flavin, 1977a; Thompson, 1982; Barra et al., 1988). This is a highly specific enzymatic system (Argaraña et al., 1980; Kumar & Flavin, 1981; Wehland & Weber, 1987a), widespread in eukaryotes and involving all known mammalian α-tubulin gene products, with the exception of a testis-specific isotype (Pratt et al., 1987). In brain tissue, newly synthesized α-tubulin normally has a carboxy-terminal tyrosine (Cleveland & Sullivan, 1985; Villasante et al., 1986; Little & Seehaus, 1988). Some mice isotypes lack this amino acid and end with a glutamyl residue. Nevertheless, the tubulin still undergoes tyrosination and detyrosination (Gu et al., 1988), the difference being that the cycle starts with Glu tubulin instead of Tyr tubulin. Despite the general occurrence of the tyrosination cycle, brain tubulin preparations obtained through classical procedures contain large quantities of a type of tubulin which cannot be tyrosinated. In vitro, under optimal conditions of tubulin tyrosination, only 50% of the protein can be tyrosinated (Barra et al., 1980; Paturle et al., 1989). Evidence has been provided for the existence of N.Tyr tubulin in vivo (Rodríguez & Borisy, 1978; Barra et al., 1980). Nevertheless, because biochemical methods to separately prepare the three types of tubulin were not available, the origin and nature of N.Tyr tubulin remained obscure. We have recently solved this problem of purification and have shown that N.Tyr tubulin had a specific epitope in the α-subunit (Paturle et al., 1989).

In the present paper, we show that non-tyrosinatable tubulin has a special primary structure and this structure is elucidated.

Materials and Methods

Materials

The buffer used for microtubule protein isolation and for phosphocellulose chromatography was 100 mM Mes [2-(*N*-morpholino)ethanesulfonic acid]/1 mM $MgCl_2$/1 mM EGTA [ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], pH 6.75 (designated MME). The sucrose/MME buffer was constructed by addition of 50% sucrose (w/v) to previously prepared MME buffer.

Phosphocellulose P11 was from Whatman. Sucrose (ultracentrifugation grade), Tris, and calcium chlorure were obtained from Merck. ATP and thermolysin were purchased

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from Boehringer Mannheim. Sepharose 4B and DEAE-Sephadex A-50 were from Pharmacia. Cyanogen bromide was from Pierce. Phosphate-buffered saline (PBS; tablets), bovine serum albumine (BSA), *O*-nitrophenyl-β-D-galactopyranoside (ONPG), Mes, EGTA, magnesium chloride, 2-mercaptoethanol, and sodium dodecyl sulfate (SDS) were purchased from Sigma.

Hydrochloric acid and acetonitrile (UV grade) were from Prolabo; sodium hydroxyde, sodium carbonate, and sodium bicarbonate were from Carlo Erba; Tween 20 (EIA grade), phosphatase alcaline conjugated antibodies, and the color development substrates were from Bio-Rad; trifluoroacetic acid (TFA) was from Applied Biosystems. The β-galactosidase-conjugated antibody was obtained from Biosys. Nitrocellulose membrane filters (BA83, 0.2 μM) were from Schleicher & Schuell, and microtiter plates for ELISA were from NUNC.

When needed, protein solutions were concentrated by ultrafiltration through centriprep 30 (Amicon).

The monoclonal Tyr tubulin antibody (clone YL $^1/_2$) was a generous gift of Dr. J. V. Kilmartin; the polyclonal Glu tubulin antibody was kindly provided by Dr. J. C. Bulinski.

Methods

Microtubule Protein Isolation. Microtubule protein from beef brain was isolated by a modification of previously published procedures (Job et al., 1985). Microtubule assembly was induced using ATP instead of GTP in the first assembly cycle. After completion of tubulin assembly, the solution was directly layered on sucrose/MME buffer and centrifuged for 2 h in a fixed-angle rotor (120 000 average, 30 °C). Pellets are stored at −80 °C for further use.

Pure tubulin was isolated from microtubule proteins by phosphocellulose chromatography in MME buffer as in Paturle et al. (1989).

Purification of Tubulin Tyrosine Ligase. Tubulin tyrosine ligase was isolated as previously described (Wehland et al., 1986) except that bovine brain instead of porcine brain was used as a starting material.

Isolation of Tubulin Isoforms. Tyr tubulin and Glu tubulin were isolated as in Paturle et al. (1989).

N.Tyr tubulin was prepared by two sequential tubulin tyrosination steps, each followed by separation of the tyrosinated and non-tyrosinated tubulin forms using affinity chromatography procedures, as described in Paturle et al. (1989).

Tubulin Proteolysis and HPLC Procedures. In order to eliminate any residual contamination by microtubule-associated proteins, N.Tyr tubulin was further purified by ion-exchange chromatography on DEAE-Sephadex A-50 according to Murphy et al. (1977). It was then concentrated and exchanged in the thermolysin buffer (50 mM Tris, 0.1 mM $CaCl_2$, pH 8 with HCl) as previously decribed (Paturle et al., 1989).

In a typical experiment, 7 mg of N.Tyr tubulin (4 mg/mL) was digested by thermolysin (1/20 w/w) for 5 h at 37 °C.

In these conditions, digestion was complete. Subsequent HPLC analysis was performed as in Eddé et al. (1990) with modifications of the elution gradients, as described in the legend to Figure 2.

Polyclonal Antibody to Non-tyrosinatable Tubulin (SK Antibody). This antibody has been described in a previous paper (Paturle et al., 1989). However, the procedures used to eliminate the immunoglobulins which recognized epitopes shared by the various tubulin subspecies were modified: homogeneous Tyr tubulin was prepared by two sequential YL $^1/_2$ immunoaffinity chromatography steps, using for the second step a newly prepared column in order to avoid a contamination by N.Tyr tubulin. The purified Tyr tubulin was coupled to Sepharose 4B according to Cuatrecasas (1970) at a concentration of 300 μg/mL of gel.

Aliquots of this gel (250 μL) were added to 5 mL of purified IgGs (100 μg/mL, in PBS containing 1% BSA). The mixture was gently rotated for 1 h at room temperature and then spun for 5 min at 10000*g*. In the present paper, the supernatant is referred to as the SK antibody. It does not react with Tyr tubulin or Glu tubulin but specifically recognizes N.Tyr tubulin.

Amino Acid Sequencing. Peptides were sequenced using a 470A gas–liquid amino acid sequencer (ABI). Fifty percent aliquots of the PTH-aa were analyzed with a 120 PTH-aa analyzer (ABI) using the standard program developed by ABI, on a C18-PTH column.

Fast Atom Bombardment (FAB). Negative FAB mass spectrometry was carried out using a ZAB-HF double-focusing mass spectrophotometer (mass range 3200 daltons at 8 keV ion kinetic energy). Spectra were recorded on a VG 11/250 data system (VG Analytical Ltd., Manchester, U.K.). The mass spectrophotometer was equipped with a saddle field atom gun (Ion Tech Ltd., Teddington, U.K.). Ionization of the sample was performed with 1 mA of 8-keV energy Xe atom beam. Each sample was dissolved in 4 μL of methanol/neutral deionized water 50/50. The matrix used was 1-thioglycerol. A total of 1 μL of matrix was first deposited on a stainless steel target and 1 μL of peptide solution was added. Mass calibration was carried out by using a saturated solution of NaI in glycerol. Wide-range single scans of small peptides (mass inferior at 2500 daltons) were produced by magnetic scanning at 8-keV accelerating voltage (scan time 12 s from 220 to 2200 amu) at a resolution of 2000 (5% valley).

Chemical Synthesis of Peptides. Peptides corresponding to the carboxy-terminal region of α-tubulin were synthesized using the standard t-Boc amino acid procedure with an automatic synthesizer (430A, ABI). For the glutamylated forms of these peptides, Glu^{445} was introduced in the sequence as Fmoc-Glu (OtBu) Obt [N^{α}-(fluorenylmethyloxycarbonyl)-glutamic acid benzotriazole ester]. After hydrolysis of the γ-*tert*-butyl ester (Roeske, 1981), the γ-carboxyl was activated as *N*-succinimidate ester which was reacted with glutamate dibenzyl ester, Glu(OBzl)α-Glu(OBzl)OBzl, to obtain the monoglutamylated γ_1 and diglutamylated $\gamma_1\alpha_2$ derivatives, respectively. After cleavage of Fmoc (Carpino & Han, 1972), synthesis was continued using the standard procedure. Peptides were then cleaved with TFA and purified by reversed-phase HPLC on a C8 column (ABI). The structure of the peptides was confirmed by amino acid sequencing and mass spectrometry.

Other Assays. Competitive ELISA tests were carried out in 96-well microtiter plates. All incubation steps were at 37 °C. In each well, 0.5 μg of non-tyrosinatable tubulin in carbonate/bicarbonate buffer (100 mM, pH 9.5) was provided. The plates were incubated for 2 h and then washed three times with PBS/0.1% Tween (PBST). SK antibody and aliquots of the fractions separated by HPLC (20 μL, C8-RP300 column; 10 μL, C18 column) or variable concentrations of synthetic peptides, in PBS/1% BSA, were mixed and preincubated for 45 min before 50 μL of the mixture was added per well. The concentration of SK antibody was kept constant at 2.5 μg/well. The plates were incubated for 1 h and washed three times with PBST before 50 μL of β-galactosidase-conjugated goat anti-rabbit IgGs (diluted 1:2000 into PBST/1% BSA) was added per well. After 1 h of incubation, the plates were washed three times with PBST and 50 μL of substrate freshly

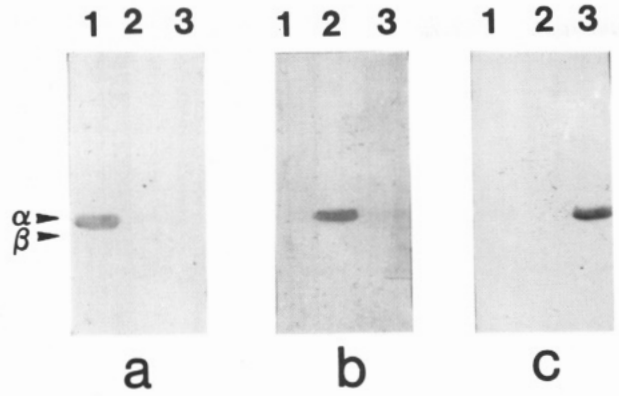

FIGURE 1: Western blot analysis of the different tubulin forms using a Tyr tubulin antibody (YL $^1/_2$) (a), a Glu tubulin antibody (b), and a N.Tyr tubulin antibody (SK antibody) (c) as probes. Equal quantities of tyrosinated tubulin (1), detyrosinated tubulin (2), and non-tyrosinatable tubulin (3) were run into 8% SDS–polyacrylamide gels, transferred onto nitrocellulose sheets, and immunoblotted with the different antibodies. The secondary antibodies were conjugated to phosphatase alcaline. The positions of α- and β-tubulin subunits are indicated.

prepared (ONPG, 0.8 mg/mL; 2-mercaptoethanol, 7 μL/mL, in PBS) was added per well. After a 30-min incubation, the reaction was stopped by addition of 50 μL of 0.5 M Na_2CO_3, pH 9.5. Absorbance was measured at 405 nm. Duplicate of triplicate assays were performed and average values are given in Table I.

Protein determination was as in Lowry et al. (1951).

Polyacrylamide gel electrophoresis and Western blots were as in Paturle et al. (1989).

RESULTS

N.Tyr Tubulin Has a Modification at the Carboxy Terminus of the α-Subunit. In agreement with previous results, Western blot analysis (Figure 1) shows that N.Tyr tubulin has a specific structure compared with normal tubulin. N.Tyr tubulin specifically reacts with a previously described polyclonal antibody (SK antibody) (Paturle et al., 1989). Furthermore, it does not react with antibodies which recognize the carboxy-terminal sites of both Tyr (Wehland et al., 1983, 1984) and Glu (Gundersen et al., 1984) tubulin. Hence, the specific antigenic determinant of N.Tyr tubulin is carried by the carboxy-terminal region of the α-subunit.

This was confirmed by thermolysin cleavage of the non-tyrosinatable tubulin followed by separation on HPLC columns of the resulting peptides (Figure 2a). The peptides were then tested for their reactivity with the SK antibody by competitive ELISA. Four reactive fractions (A–D) were observed.

Fraction A peptide(s) reacted weakly, showing a low specific activity. This reactivity was not assayable during subsequent purification steps and fraction A was not further analyzed.

Reactive peptides contained in fractions B, C, and D were further purified using HPLC (Figure 2b–d). This yielded six peaks (1–6). The amount of material contained in peak 5 was unsufficient to obtain an amino acid sequence. Peptides corresponding to peaks 1–4 were found to have the same amino acid sequence, VEGEGXEEG. This was found to match the carboxy-terminal region of several α-tubulin isogene products (Figure 3) (Little & Seehaus, 1988). This confirms that the SK antibody recognizes the carboxy-terminal domain of N.Tyr tubulin. The above sequence contains a gap noted X. Previous work has shown that this gap can be attributed to α-tubulin glutamylation at position 445 (Eddé et al., 1990).

The peptide corresponding to peak 6 is an apparent and interesting exception. Its amino acid sequence VGEGMEEG

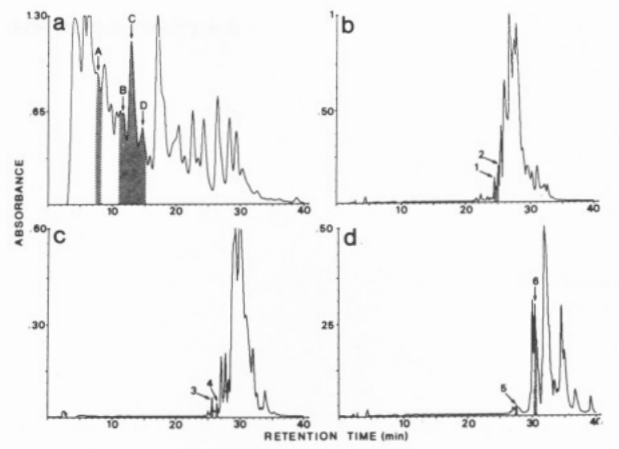

FIGURE 2: HPLC purification of the peptides obtained by thermolysin digestion of N.Tyr tubulin. The peptide mixture was injected in a C8-RP300 reversed-phase column (10 × 250 mm, Brownlee) at 95% A/5% B and at a flow rate of 4 mL/min. The elution gradient was linearly increased from 95% A/5% B to 50% A/50% B in 50 min (a). The eluted fractions were evaporated under speed vacuum and resuspended in 400 μL of water, and aliquots were tested for their reactivity with the SK antibody by indirect competitive ELISA. Reactive fractions B, C, and D were further chromatographed on a C18 column (4.6 × 220 mm) (b, c, and d, respectively) at a flow rate of 1 mL/min. The gradient was linearly increased from 95% A/5% B to 71% A/29% B in 40 min. In these latter chromatographies, peaks were collected manually and were tested for their reactivity with the SK antibody as above. Hatched areas refer to the reactive fractions. Solution A was 0.1% TFA in water, and solution B was 0.09% TFA and 70% acetonitrile in water. Absorbance was monitored at 215 nm.

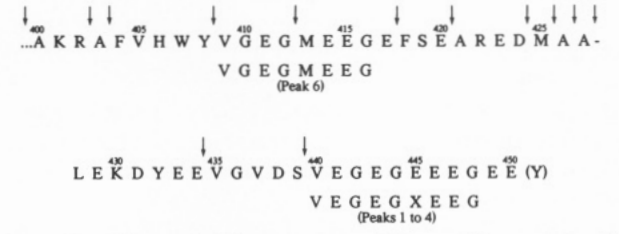

FIGURE 3: Alignment of the sequences of the peptides contained in peaks 1–4 and in peak 6 (cf. Figure 2) with the sequence of pig α-tubulin (Ponstingl et al., 1981). Arrows indicate potential thermolysin cleavage sites.

was found to match the region located between amino acid residues 409–416 of known α-tubulin sequences (Figure 3). The reactivity of this peptide is discussed below.

Combination of Peptide Sequence Analysis and Mass Spectrometry Strongly Suggests That N.Tyr Tubulin Lacks a Glutamyl Group at Position 450, on the α-Subunit. The peptide sequences (peaks 1–4) terminate at a position corresponding to residue 448 of the α-tubulin chain (Figure 3). At this stage, we do not know if this is the real end of the peptides. Sequence determination is stopped at this position due to the repetitive sequence of Glu and Gly at the proximity of the carboxy terminus.

Further analysis of the structure was carried out using mass spectrometry (Figure 4).

The accurate masses of the carboxy-terminal peptides (peaks 1–4) presented increments of 129 daltons or of a multiple of 129 daltons, as compared with their masses calculated from the basic amino acid sequence VEGEGXEEG. Since 129 daltons is the mass of a glutamyl group, these results show that peptides corresponding to peaks 1–4 contained 3, 4, 5, and 6 additional glutamyl residues, respectively.

There are several possible locations of these additional residues. In the case of peak 1, one possibility would be that

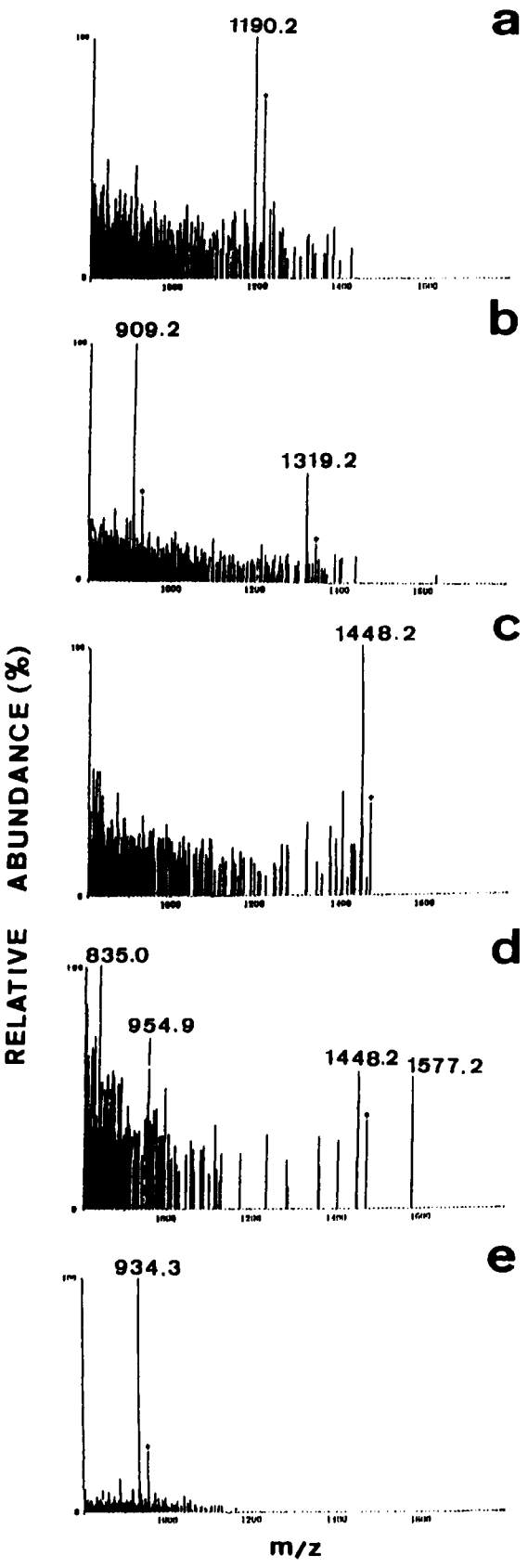

FIGURE 4: Mass spectra of thermolysin peptides reactive to the SK antibody. 20% aliquots of peaks 1 (a), 2 (b), 3 (c), 4 (d), and 6 (e) were analyzed by negative FAB mass spectrometry. Ionization is performed by loss of a proton. The peaks correspond therefore to M-1. Sodium adducts are observed for almost all molecular ions and are indicated by an asterisk. They correspond to $(M + Na - H)^-$.

Table I: Immunoreactivity of Some Synthetic Peptides with the SK Antibody As Determined by Indirect Competitive ELISA

	peptides	concn for 50% competition with N.Tyr tubulin[a]	
a	V E G E G E E E G E E Y	none	*(1)*
b	V E G E G E E E G E E	none	*(1)*
c	V E G E G E E E G E E E	none	*(2)*
d	V E G E G E E E G E E E E	none	*(2)*
e	V E G E G E E E G E E E E E	none	*(2)*
f	E E G E	100 μM	
g	V E G E G E E E G E	6.6 μM	
h	V E G E G E E E G E E	2 μM	
i	V E G E G E E E G	none	*(1)*

[a] The numbers in parentheses are defined as follows: 1, the highest concentration tested (160 μM) gave no competition; 2, the highest concentration tested (160 μM) did not give 30% competition.

the peptide corresponds to the normal sequence of the carboxy-terminal region of detyrosinated α-tubulin: ^{440}VEGEGEEEGEE^{450} (the additional Glu residues have been underlined). However, as stated above, it is most likely that the gap observed in the sequence at position 445 reflects the presence of a lateral glutamyl chain at this position. In this case, the peptide would not extend up to the position 450 but would be shorter, ending at position 449 or 448, depending on the number of glutamyl units present in the lateral chain. According to this interpretation, the increased glutamyl content of peaks 2–4 would be related to the extension of the lateral chain.

Analysis of the peak 6 peptide strongly indicated that the carboxy-terminal peptides (peaks 1–4) actually stop at Glu^{449}. For this peptide, observed and calculated masses differ by one glutamyl residue and Figure 3 shows that this residue must occur at position 417. The complete sequence of peptide 6 is therefore ^{409}VGEGMEEGE^{417}. Remarkably, the terminal ^{414}EEGE^{417} sequence matches the carboxy-terminal region of α-Glu tubulin from position 446 to position 449. Because the EEGE sequence is recognized on the digested but not on the undigested α-tubulin, then it is likely that the SK antibody recognizes the sequence EEGE at a carboxy-terminal end. In the present case, it was fortunate that this sequence was just next to a thermolysin cleavage site, thus revealing the structure of the antibody-specific epitope. Taken together these results strongly suggest that N.Tyr tubulin has an α-subunit which is truncated at position 449.

However, this conclusion relies on the assumption that the sequence EEGE is absolutely required for reactivity with the SK antibody. Tests of this assumption are shown below.

Indirect Competitive ELISA Analysis Confirms That the α-Subunit of N.Tyr Tubulin Ends at Position 449. Peptides corresponding to different possible structures from position 440 to the α-tubulin carboxy terminus were synthesized and tested for their reactivity with the SK antibody. As expected, the peptides corresponding to normal Tyr and Glu tubulin (Table I, rows a–b) did not react with the antibody. Similar peptides with an additional branched polyglutamyl chain did not react either (Table I, rows c–e). This shows that such a modification of the tubulin structure cannot account for the reactivity of peptides 1–4 with the SK antibody (Figure 2). A positive reaction occurs when the carboxy terminus ends

Table II: Primary Polypeptide Structures As Determined from the Combination of Sequence Analysis, Mass Spectrometry, and Competitive ELISA Results

Peak 1	V E G E G E E E G E E
Peak 2	V E G E G E E E G E E E
Peak 3	V E G E G E E E G E E E E
Peak 4	V E G E G E E E G E E E E E
Peak 6	V G E G M E E G E

with EEGE. The EEGE peptide itself reacts with the antibody. This reactivity is greater when the sequence forms part of a peptide reproducing larger parts of the α-tubulin carboxy-terminal region (Table I, rows g and h). A peptide terminating at position 448 did not react (Table I, row i), and this rules out the possibility that peptides 1–4 terminate at this position. These results demonstrate the high specificity of the SK antibody and show that peptides 1–4 terminate by EEGE. The complete primary structures of the thermolysin peptides are given in Table II.

Discussion

These results show that non-tyrosinatable tubulin, as recognized by its interaction with the SK antibody, is a well-defined variant of brain tubulin. It differs from the tyrosinated form of tubulin by the absence of the last two amino acids at the carboxy terminus of the α-subunit. The principal questions which arise concern other forms of N.Tyr tubulin that might exist, the relationship between the modification reported here and the lack of reactivity with tubulin tyrosine ligase, and the origin of the reported variant.

N.Tyr tubulin could be a mixture of several different forms of modified tubulin, of which only one form reacts with the SK antibody. Our results do not favor this possibility. The purification procedures are the same for the peptides 1–4, which contain the carboxy-terminal region which reacts with the SK antibody, and for peptide 6. The latter forms part of a highly conserved region of α-tubulin (Little & Seehaus, 1988) and will therefore arise from all isoforms of N.Tyr tubulin. Although there is a potential thermolysin cleavage site (Matsukara et al., 1966; Morihara, 1967) in the midregion of the sequence, peptide 6 is apparently not cleaved during enzymatic proteolysis: we have varied digestion times from 4 to 24 h without observing any variation in the HPLC profiles. Its HPLC peak area can thus be used for calibration to determine the ratio of the amount of carboxy-terminal peptides recognized by the SK antibody to the total expectable amount of carboxy-terminal peptides.

The determined ratio of the total amount of peptides 1–4 to peptide 6 was 1.5, and this corresponds to a stoichiometry close to 1. This indicates that in these experiments nearly all of the N.Tyr tubulin reacted with the SK antibody.

N.Tyr tubulin lacks an amino acid at the site of action of tubulin tyrosine ligase and this provides a satisfactory explanation as to why it cannot be tyrosinated. The present study does not prove that the absence of a glutamate residue at position 450 leads to loss of tubulin reactivity with the tubulin tyrosine ligase. This would require the addition of a glutamate residue at position 450 of N.Tyr tubulin together with a renewed activity as a substrate in the tyrosination cycle.

The series of reactions which transform normal α-tubulin into its non-tyrosinatable form is unclear. One possibility is the action of carboxypeptidases in the following sequence: Tyr tubulin → Glu tubulin → N.Tyr tubulin. Although this is the most obvious pathway to N.Tyr tubulin, other mechanisms may be preferred. Tubulin carboxypeptidase, the enzyme which converts Tyr tubulin into Glu tubulin, stops at Glu^{450} (J. Wehland, unpublished results). Other known tissue carboxypeptidases either react with carboxy-terminal basic residues or are of the carboxypeptidase A type, an enzyme which is unable to remove the glutamate residue at position 450 even after very long incubations (Raybin & Flavin, 1977).

Another possibility is that the enzyme responsible for the formation of N.Tyr tubulin is a dipeptidyl carboxypeptidase of the kinase II or converting enzyme type. This enzyme is present in the brain and is known to be involved in the processing of opioid enkephalin peptides (Stewart et al., 1981). Its substrate specificity is such that it should be capable of removing the carboxy-terminal glutamyl-tyrosine dipeptide. The reaction would then stop because this enzyme does not react with peptides having a glutamate at the carboxy-terminal position. In this case, N.Tyr tubulin could be generated from Tyr tubulin but not from Glu tubulin. Another brain enzyme, neutral endopeptidase, could also catalyze the formation of N.Tyr tubulin (Roques et al., 1991). However, these peptidases are not known to be active on large proteins, while massive amounts of normal tubulin are transformed into N.Tyr tubulin. This is in favor of the existence of a tubulin-specific peptidase, different from tubulin carboxypeptidase, and catalyzing the formation of N.Tyr tubulin.

The answer to these questions should be readily obtained in the near future by standard biochemical procedures.

Another crucial question is the reversibility of the reaction which leads to N.Tyr tubulin. It would be fascinating if an enzyme analogous to tubulin tyrosine ligase, but transferring a glutamyl-tyrosine group, existed. This question should be simple to answer since biochemical amounts of N.Tyr tubulin are available and may be tested as substrates in reactions of this type.

The answers to these basic questions will limit the assumptions which can be made concerning the physiological significance of N.Tyr tubulin. For example, the relative abundance of Tyr tubulin and Glu tubulin reflects to some extent the dynamic of the microtubular networks, since Glu tubulin tends to accumulate in stable, less dynamic microtubules (Bré et al., 1987; Khawaja et al., 1988; Kreis, 1987; Schulze et al., 1987; Wehland & Weber, 1987b; Baas & Black, 1990). If a sequence Tyr tubulin → Glu tubulin → N.Tyr tubulin is responsible for the appearance of non-substrate tubulin, then it could be that the accumulation of this variant would reflect the presence of very long lived polymers. If, on the contrary, N.Tyr tubulin is generated directly from Tyr tubulin in a reaction incompatible with a Glu tubulin intermediate, its presence might be unrelated to microtubule stability. Similarly, the abundance of N.Tyr tubulin, which seems to be developmentally regulated (Rodríguez & Borisy, 1978; Barra et al., 1980), will have a totally different significance depending on the reversibility of the reaction.

N.Tyr tubulin does not result from an unphysiological digestion of tubulin during the preparative procedures. An immunoblot of a sample prepared by SDS lysis of the brain from a freshly killed rat gave a positive reaction with the SK antibody (data not shown). Recent immunofluorescence results obtained in this laboratory show that this isoform occurs in neuronal primary cell cultures. However, the enzyme(s)

responsible for the generation of N.Tyr tubulin might be active during tubulin purification and we do not yet know if the relative in vivo abundance is the same as that in vitro.

Despite its widespread occurrence in eukaryotes and the highly specific enzymatic system which it involves, the functional significance of the tyrosination–detyrosination cycle of tubulin has not yet been elucidated. N.Tyr tubulin represents a large proportion of brain tubulin which is withdrawn from this cycle. If the ways that this might effect brain functions were known, insights migh be gained concerning the significance of the tyrosination–detyrosination cycle.

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