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Class I and IVa β-tubulin isotypes expressed in adult mouse brain are glutamylated

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Abstract Several types of post-translational modifications contribute to the high level of tubulin heterogeneity in the brain. An important modification is glutamylation of the major brain-specific isotypes, such as class Ia/b of α-tubulin and classes II and III of β-tubulin. Here we describe experiments to determine if additional, minor tubulin isotypes, expressed in adult mouse brain, could also be glutamylated. Purified tubulin from adult mouse brain was cleaved with thermolysin. Proteolytically released carboxy-terminal peptides of both α- and β-tubulin were isolated by sequential anion exchange and reverse-phase column-chromatography. Anionic peptides were then characterized by amino acid sequencing and mass spectrometry. We show that brain-specific class IVa and constitutive class I β-tubulin isotypes can be glutamylated, at Glu^{434} and Glu^{441}, respectively.

Key words: Glutamylation; Tubulin isotype; Mass spectrometry; Post-translational modification

1. Introduction

Brain tubulin exhibits a high degree of heterogeneity up to 21 isoforms having been resolved by IEF [1]. This heterogeneity is due both to differential expression of α- and β-tubulin genes, producing 5 α- and 6 β-tubulin isotypes in mammals [2,3], and to subsequent post-translational modifications of these isogene products (for review see [4]). For α-tubulin, the following post-translational modifications have been described: reversible acetylation of Lys^{40} [5], removal and re-addition of the C-terminus Tyr^{451} [6], removal of the Glu^{450} after detyrosylation [7], and polyglutamylation of Glu^{445} [8]. For β-tubulin, it has been reported that the neuron-specific class III isotype can be phosphorylated at Ser^{444} [9,10] or Tyr^{437} [11], and polyglutamylated at Glu^{438} [12]. The brain-specific class II isotype can also be glutamylated at Glu^{435} [13,14].

With the exception of acetylation, these different post-translational modifications occur in the carboxy-terminal domain of both α- and β-tubulins, and more precisely in the hypervariable region [15], which extends, for α-tubulins from residue 440 to 451, and for β-tubulins from 431 to 445. Most of the heterogeneity between the different isotypes is localized in these regions [16] which are rich in acidic amino acid residues. Thus glutamylation increases the acidity of an already highly acidic domain of tubulins. Futhermore, this negatively-charged carboxy-terminal tail of the molecules is exposed at the outer surface of the microtubule, and is known to be functionally important. It is involved in the interaction with various MAPs including structural [17–19] and motor proteins [20–22]. Such interactions could regulate both the dynamics of tubulin assembly into microtubules and microtubule-mediated transport of various cellular components [20,23–25]. Furthermore, this acidic carboxy-terminal sequence could modulate the interaction with Ca^{2+}, an inhibitor of tubulin assembly [26].

The aim of this work was to determine if minor β-tubulin isotypes expressed in adult mouse brain could also be modified by post-translational polyglutamylation. Previous studies have reported that digestion of mouse brain tubulin dimers by thermolysin releases the peptide ^{440}VEGEGEEEGEEY^{451} from the carboxy-terminus of isotypes ma1/2 [8,27]. In the present paper we show that this treatment also releases carboxy-terminal peptides of other tubulin isotypes expressed in adult mouse brain. These peptides were characterized both by sequence analysis and mass spectrometry. We demonstrated that the brain specific class IVa β-tubulin isotype (gene mβ4) and the constitutive class I β-tubulin isotype (gene mβ5) can be glutamylated at Glu^{434} and Glu^{441}, respectively.

2. Materials and methods

2.1. Chemicals

The following were purchased: 1,4-piperazine diethanesulfonic acid (Pipes) and guanosine 5'-triphosphate (GTP) (Boehringer-Mannheim); Tris (hydroxymethyl)-aminomethan (Merck); Ethylene glycol bis (β-aminoethyl ether)-*N,N,N',N'*-tetra acetic acid (EGTA) (Sigma); Acetonitrile (Baker) and trifluoroacetic acid (TFA) (Applied Biosystem). Taxol purified from Taxus baccata was a gift from Dr. D. Guénard (ICSN, Gif-sur-Yvette, France).

2.2. Tubulin purification

Tubulin was prepared from adult mice (Swiss-IFFA CREDO) brains by taxol-induced microtubule assembly, according to Vallee et al. [28]. To determine the concentration of tubulin the method of Bradford was used [29].

2.3. Proteolytic digestion

Tubulin (2 mg/ml in 0.1 mM Pipes, 2 mM EGTA, 1 mM $MgCl_2$, pH 6.8) was digested with thermolysin (Boehringer Mannheim), in thermolysin-buffer (50 mM Tris-HCl buffer, pH 8, containing 0.1 mM $CaCl_2$) with 1:20 enzyme/substrate (w/w), for 5 h at 37°C. The reaction was stopped by freezing the sample at −80°C. Before injection on the HPLC column, the thermolysin digestion products were centrifuged at 12,000 × *g* for 2 min.

2.4. HPLC separation

Purification of the carboxy-terminal acidic peptides of α- and β-tubulin was performed by anion exchange chromatography followed by

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Abbreviations: DEAE, diethylaminoethyl; IEF, isoelectro focusing; HPLC, high-performance liquid chromatography; L-SIMS, liquid second ion mass spectrometry; MAPs, microtubule associated proteins; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid.

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reverse-phase chromatography on a Waters (Millipore) HPLC apparatus. The elution was monitored at 214 nm.

The DEAE column (DEAE 5PW, Protein Pack, 75 × 7.5 mm, Waters) was eluted with a linear gradient from 0 to 100% of buffer B in 60 min at a flow rate of 1 ml/min. Buffer A was 20 mM Tris-HCl, pH 8.0, 50 mM NaCl, and buffer B was 20 mM Tris-HCl, pH 8.0, 500 mM NaCl. The fractions eluted from the DEAE column were further purified by reverse-phase HPLC.

The reverse-phase column (C-18, 5 μm, Brownlee, 220 × 2.1 mm) was eluted at 15°C at a flow rate of 200 μl/min. Solvent C was deionized water and solvent D was 80% acetonitrile in water. These two solvents were acidified to pH 3 with trifluoroacetic acid. The fractions collected from the DEAE column were eluted with a step-gradient of solvent D (80% v/v acetonitrile, pH 3) as follows: 1% D in C for 10 min, then from 1 to 10% for 1 min, and finally from 10 to 45% in 35 min. Before injection, the pH of each sample was adjusted to pH 3.0 with TFA.

2.5. Amino acid sequencing

Peptides were sequenced by automated Edman degradation using a 470A gas-liquid sequenator (Applied Biosystems) with the modification described by Le Caer and Rossier [30].

2.6. Mass spectrometry

L-SIMS analysis was carried out on an Autospec 6F mass spectrometer (Fison, Manchester, UK). Spectra were generated by a 25 keV cesium ion beam. Cesium iodide clusters were used for mass calibration. The sample matrix was a 1:1 mixture of glycerol and thioglycerol. The spectra resulted from averaging ten scans/spectra with a resolution of 2000. Methylation of free carboxylic groups was performed as described by Redeker et al. [27] except that about 20–100 pmol of peptide was used in each experiment. 1 μl of this solution was mixed with 1 μl of the matrix directly on the target and analyzed immediately.

3. Results

3.1. Digestion of purified brain tubulins, and separation of carboxy-terminal peptides

After purification by taxol-induced microtubule assembly, brain tubulin was digested by thermolysin. The completion of the enzymatic digestion was verified by SDS-PAGE analysis (results not shown). Since the cleavage-specificity of thermolysin is broad [31], a large number of peptides were generated by hydrolysis with this enzyme. To isolate the acidic carboxy-terminal fragments of tubulins, peptide products were separated on a DEAE column (Fig. 1). Previous studies have reported that, under these conditions, the acidic carboxy-terminal peptides of both α- and β-tubulin were eluted after 25 min and that peaks DEAE-0 to -3 contained peptides of isotypes ma1/2 unmodified, mono, bi and triglutamylated, respectively [8,27]. Peaks DEAE-0 to -7 were collected as separate fractions and further purified by reverse-phase HPLC. Individual carboxy-terminal peptides were subsequently characterized by sequence analysis and mass spectrometry. The results of the identification of the different peptides eluted in fractions DEAE-0 to DEAE-7 are presented in Table 1.

3.2. Glutamylation of the constitutive class I β-tubulin isotype

The reverse-phase elution profile of peak DEAE-0 is shown in Fig. 2, panel A. Three peaks were resolved and denoted as RP-1 to RP-3. The amino acid sequence obtained for the peptide eluted under peak RP-3 was FGEEAXEE---. This sequence matches the carboxy-terminal sequence of class I β-tubulin isotype (gene mβ5), from residues 436 to 443, where X indicates that no signal corresponding to Glu^{441} was detected. It has been reported previously that such a gap in the sequence of carboxy-terminal tubulin peptides could be the result of glutamylation of the γ-carboxylic group of a glutamyl residue [8]. To confirm the nature of the modification and to determine the number of glutamyl units post-translationally added to Glu^{441}, the mass of the peptide was measured by L-SIMS (Fig. 3, panel B). The ion at mass/charge ratio (*m/z*) 1237.2 corresponds to the carboxymethylated peptide ^{436}FGEEAEEEA^{444}, with an additional mass of 143 Da, which is the mass of a carboxymethylated glutamyl unit. Thus it is proposed that the corresponding peptide is monoglutamylated. No higher degrees of glutamylation for this class I β-tubulin isotype at position Glu^{441} have been found in the other DEAE peaks. This suggests that higher degrees of glutamylation of this class of tubulin do not exist at a detecTable level, in our conditions, in the murine brain.

Fractions RP-1 and -2 (Fig. 2, panel A) contained carboxy-terminal peptides of ma1/2 tubulin isotypes, starting at amino acid 440 (Table 1). The peptide eluted under peak RP-1 extended to amino acid 449 and bore a glutamyl residue added post-translationally on Glu^{445}, whereas the peptide eluted under peak RP-2 extended to amino acid 450 and was unmodified [7,32]. Both of these peptides had the same mass, the mass-spectrum of the carboxymethylated peptide eluted as peak RP-2 being shown in Fig. 3, panel A. Thus, these two peptides differed by the position of a single glutamyl residue.

3.3. Glutamylation of the brain-specific class IVa β-tubulin isotype

The reverse-phase elution profile of fraction DEAE-4 is

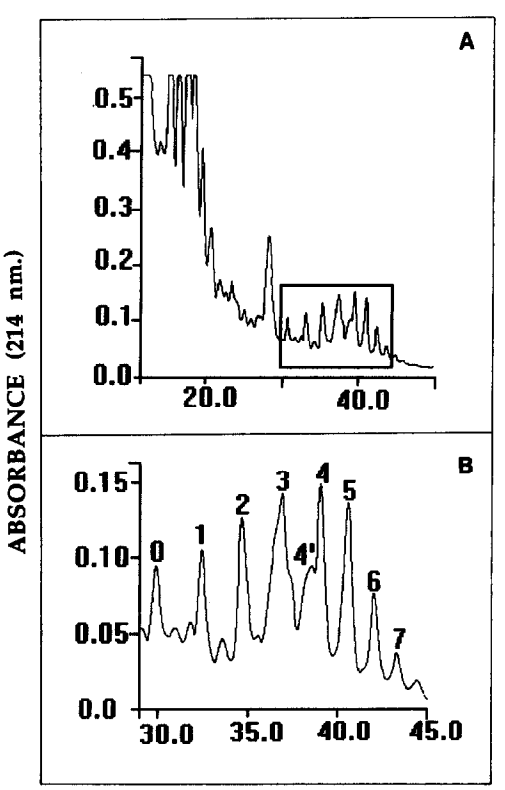

Fig. 1. Purification by DEAE HPLC of the tubulin carboxy-terminal peptides obtained after thermolysin treatment. The complete elution profile from cleaved tubulins is shown in panel A, and the boxed region is shown enlarged in panel B. Fractions, denoted DEAE-0 to DEAE-7, were collected separately for further purification.

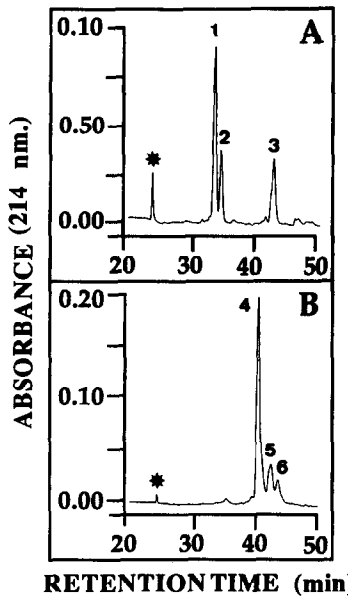

Fig. 2. Reverse-phase HPLC purification of the fractions of tubulin fragments collected from the DEAE column. The elution profile of fractions DEAE-0 and DEAE-4 are shown in panels A and B, respectively. Fractions were denoted RP-1 to RP-6. The peak denoted by * results of the rapid increase of the solvent B concentration at the beginning of the gradient.

shown in Fig. 2, panel B. Three peaks were resolved and denoted RP-4 to RP-6. Fractions RP-5 and RP-6 each contained a peptide with the sequence AEEGXFEEEA---, which matches the carboxy-terminal sequence of class IVa β-tubulin (isotype mβ4), from residues 430 to 439. No signal was detected for the cycle corresponding to Glu^{434}. As it was assumed, this position is probably modified by addition of one or more glutamyl unit. By mass spectrometry, the same molecular mass for these two peptides was obtained. The mass spectrum of the peptide eluted as fraction RP-5 is shown in Fig. 3, panel D. The ion at m/z ratio 1952.5 corresponds to the carboxymethylated peptide ^{430}AEEGEFEEEAEEE^{442}, with addition of two units of 143 Da. Thus, the peptide appeared to be biglutamylated. This result shows that class IVa β-tubulin isotype can be glutamylated, and that the two peptides eluted as fractions RP-5 and RP-6 have been post-translationally modified by the addition of two glutamyl residues.

The two peptides could differ by the linkage between the two glutamyl groups added post-translationally on to Glu^{434}. The second additional glutamyl unit can be linked by an amide bond from either the α- or the γ-carboxyl group of the first glutamyl unit added. However, the existence of two sites of glutamylation, Glu^{434} and Glu^{440}, each modified by the addition of one glutamyl unit cannot be excluded since the last three residues (from Glu^{440} to Glu^{442}) could not be obtained by sequencing. According to this latter hypothesis, one of the fractions, RP-5 or RP-6, could contain a peptide biglutamylated at Glu^{434} and the other peak a peptide modified at both positions, Glu^{434} and Glu^{440}, each by one glutamyl unit.

Carboxy-terminal peptides of class IVa β-tubulin were also identified in fractions DEAE-2, -3, -5 and -6 bearing no, one, three and four additional glutamyl units respectively (see Table 1).

The peptide eluted as fraction RP-4 matched the carboxyl terminus of the class II β-tubulin isotype, from amino acids 430 to 443, bearing two glutamyl units added post-translationally (mass spectrum shown in Fig. 3, panel C). This confirms results reported previously for polyglutamylation of class II β-tubulin isotypes [13,14].

4. Discussion

In this study, the different tubulin isotypes expressed in adult mouse brain, with the exception of class III β-tubulin, were isolated. Glutamylation of the major brain-specific tubulin isotypes previously described was observed, and we have in addition characterized glutamylation of class I and IVa β-tubulin isotypes. We have shown that the brain-specific β-tubulin isotype class IVa (gene mβ4) and the constitutive β-tubulin isotype class I (gene mβ5) can be glutamylated, at Glu^{434} and Glu^{441} respectively. Expression levels of these two β-tubulin isotypes in mouse brain are minor compared to those of class II and III β-tubulin isotypes (see Table 1), [33,34]. Thus glutamylation, even of these minor β-tubulin isotypes, contributes a major part, over the other reported post-translational modifications, to the high heterogeneity of brain tubulins [8]. Recently, immunoblotting studies using a monoclonal antibody (GT335) recognizing glutamylated tubulin [35,36] have suggested that the high heterogeneity of axonemal tubulin from mammalian spermatozoa is partially correlated with post-translational polyglutamylation. It was proposed that the major testis-specific mα3/7, class IVb (mβ3) and the constitutive class I (mβ5)

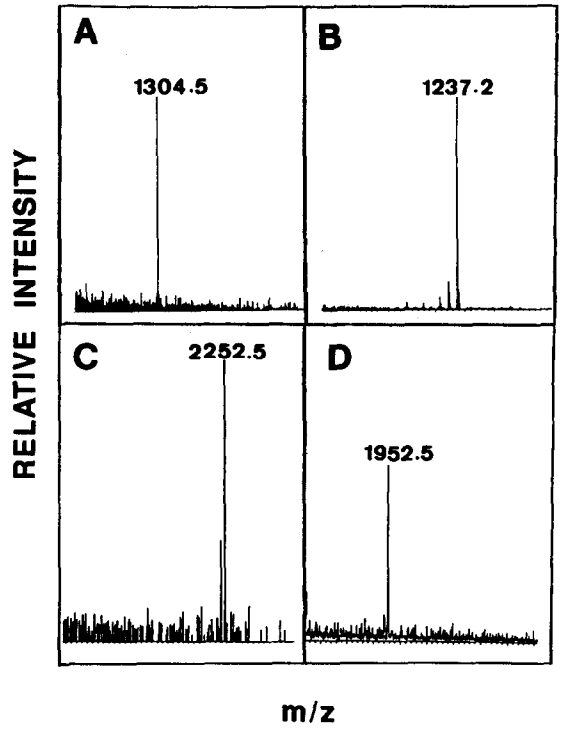

Fig. 3. Mass spectrometry analysis of carboxymethylated tubulin carboxy-terminal peptides. Monoisotopic *m/z* values of the protonated molecular ions (MH^+) are indicated. Spectra of carboxymethylated carboxy-terminal peptides: unmodified mα1/2 isotype (fraction RP-2), mβ5 bearing one additional glutamyl unit (RP-3), and mβ2 (RP-4) and mβ4 (RP-5), each bearing two additional glutamyl units, are shown in panels A, B, C and D, respectively.

Table 1
Sequence and mass (MH^+) obtained for the different peptides contained in fractions DEAE-0 to −7

DEAE peaks	Sequence	Mass MH^+	Structure	Localization in the molecule	%
0	VEGEGXEEG--	1304.5	VEGEGEEEGE E	mα1/2 (440-449)	53
	VEGEGEEEG--	1304.4	VEGEGEEEGEE	mα1/2 (440-450)	21
	FGEEAXE--	1237.2	FGEEAEEEA E	mβ5 (436-444)	26
1	VEGEGXEEG--	1447.3	VEGEGEEEGEE E	mα1/2 (440-450)	100
2	VEGEGXEEG--	1590.5	VEGEGEEEGEE $(E)_2$	mα1/2 (440-450)	86
	AEEGEFEEEA--	1666.7	AEEGEFEEEAEEE	mβ4 (430-442)	14
3	VEGEGXEEG--	1733.4	VEGEGEEEGEE $(E)_3$	mα1/2 (440-450)	59
	VEGEGXEEG--	1610.5	VEGEGEEEGEEY E	mα1/2 (440-451)	9.3
	ADEQGXFEEEEG--	2109.4	ADEQGEFEEEEGEDEA E	mβ2 (430-445)	12.8
	AEEGXFEEEA--	1809.4	AEEGEFEEEAEEE E	mβ4 (430-442)	18.9
4'	VEGEGXEEG--	1753.4	VEGEGEEEGEEY $(E)_2$	mα1/2 (440-451)	100
4	ADEQGXFEEEEG--	2252.5	ADEQGEFEEEEGEDEA $(E)_2$	mβ2 (430-445)	75.8
	AEEGXFEEEA--	1953.5	AEEGEFEEEAEEE $(E)_2$ or AEEGEFEEEAEEE E E	mβ4 (430-442)	24.2
5	ADEQGXFEEEEG--	2395.8	ADEQGEFEEEEGEDEA $(E)_3$	mβ2 (430-445)	55.6
	VEGEGXEEG--	1896.5	VEGEGEEEGEEY $(E)_3$	mα1/2 (440-451)	22.2
	AEEGXFEEEA--	2095.5	AEEGEFEEEAEEE $(E)_3$ or (with m+n=3) AEEGEFEEEAEEE $(E)_m$ $(E)_n$	mβ4 (430-442)	22.2
6	ADEQGXFEEEEG--	2538.8	ADEQGEFEEEEGEDEA $(E)_4$	mβ2 (430-445)	74.1
	VEGEGXEEG--	2039.4	VEGEGEEEGEEY $(E)_4$	mα1/2 (440-451)	13
	AEEGXFEEEA--	2238.4	AEEGEFEEEAEEE $(E)_4$ or (with o+p=4) AEEGEFEEEAEEE $(E)_o$ $(E)_p$	mβ4 (430-442)	12.9
7	ADEQGXFEEEEG--	2681.5	ADEQGEFEEEEGEDEA $(E)_5$	mβ2 (430-445)	100

Localisation in the molecule refers to the sequences of tubulin isotypes published by Sullivan [38]. The relative amount (%) of each carboxy-terminal tubulin peptide was calculated by dividing by the total amount of peptide eluted in each DEAE fraction.

tubulin isotypes are glutamylated. Our results indicate that the constitutive isotype class I can be polyglutamylated in adult mouse brain, and we have specified the site where this modification occurs.

Since the original discovery of glutamylation of mα1/2 tubulin isotypes at Glu^{445} [8], several other isotypes have been found to be glutamylated. Alexander et al. [12] have shown that the neuron-specific class III β-tubulin isotype from adult bovine brain can be polyglutamylated at Glu^{438}. Redeker et al. [13] and Rüdiger et al. [14] have reported that the major neuron-specific class II β-tubulin isotype from pig brain can be polyglutamylated at Glu^{435}. Together with our present observations, it thus appears that all β-tubulin isotypes expressed in mouse brain can be glutamylated.

In Table 2 the primary sequences of the glutamylated carboxy-terminal tubulin peptides described to date have been

Table 2
Comparison of the carboxy-terminal sequences of the different α- and β-tubulin isotypes known to be glutamylated

			-1 0 +2
mα1/2		438	V D S V E G E G **E** E E G E E Y
mβ2	classII	427	D A T A D E Q G **E** F E E E E G E D E A
mβ3	classIVb	427	D A T A E E E G E F E E E A E E E V A
mβ4	classIVa	426	Q D A T A E E G **E** F E E E A E E E V A
			or
		432	E G **E** F E E E A E E E V A
mβ5	classI	433	E E D F G E E A **E** E E A
bβ2	classIII	430	A E E E G E M Y **E** D D E E S E A Q G P K

m: mouse, b: bovine. Experimentally verified, modified glutamyl residues are shown in bold letters. A pentapeptide homologous between all of the sequences is underlined.

aligned at their respective modified glutamyl residue. In the α-tubulin (ma1/2), class II (mβ2) and IVa (mβ4) β-tubulin the modified residues are sequentially equivalent to one another, as shown by the homologous pentapeptides (underlined) that each contain the modified residue. Using this homology we can predict, for example that Glu^{435} of the closely-related class IVb β-tubulin (mβ3) is likely glutamylated. However, in the class I β-tubulin (mβ5) the equivalent residue at a first site (underlined) is not glutamyl, but Asp^{435} (Table 2) and another glutamyl residue, at a second site further along the sequence (Glu^{441}) is found to be modified. By homology with this latter experimentally identified site, Glu^{440} in the class IVa β-tubulin may be expected to be modified, in addition to Glu^{434}. Furthermore, in the class III β-tubulin (bβ2) (Table 2) Glu^{435} is not modified, whereas the subsequent glutamate in the sequence (Glu^{438}) is as reported by Alexander et al. [12]. Overall this represents a complicated situation which renders analysis and prediction of the site(s) of glutamylation difficult. For now, it would seem that the enzyme or enzymes responsible for glutamylation recognise locally similar, rather than globally homologous subtrates. Nevertheless, in each case it appears that the enzyme is able to specifically recognize a particular glutamyl residue. Thus, from the alignment of experimental positions shown in Table 2, relative to the modified glutamyl (position 0) residues at position −1 are hydrophobic and usually Ala or Gly, and are acidic (Glu or Asp) at position +2. Clearly however, these restrictions are insufficient by themselves to determine substrate-specificity.

Glutamylation modifies the carboxy-terminal region of tubulin, which is implicated in interactions between microtubules and MAPs. Kotani et al. [37] have suggested that binding of MAPs to microtubules is based upon electrostatic as well as hydrophobic interactions. Thus by increasing the negative charge in this region, polyglutamylation could modulate affinity of MAPs for the carboxy-terminal region of tubulin isotypes, and thereby influence the stability of the microtubule.

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