FEBS Letters 459 (1999) 90–94 FEBS 22667

Synthetic peptides identify the minimal substrate requirements of tubulin polyglutamylase in side chain elongation

Stefan Westermann, Uwe Plessmann, Klaus Weber*

Max Planck Institute for Biophysical Chemistry, Department of Biochemistry, Am Fassberg 11, 37077 Goettingen, Germany

Received 26 August 1999

Abstract The minimal sequence requirement of *Crithidia* tubulin polyglutamylase is already fulfilled by tubulin-related peptides carrying a free α-carboxylate on a glutamic acid residue. Since the product of each glutamylation step fulfills the substrate requirements necessary for the next cycle, very long side chains are generated with brain tubulin as a substrate. Up to 70 mol of glutamic acid was incorporated per αβ-heterodimer. We speculate that the strict choice of a particular glutamate residue for the formation of the isopeptide bond initiating a novel side chain is made by a tubulin monoglutamylase which requires the entire tubulin as substrate.

Key words: Polyglutamylation; Trypanosomatid; Post-translational modification; Tubulin; *Crithidia fasciculata*

1. Introduction

The acidic carboxy-terminal region of tubulins, which is not revealed in the atomic model of the αβ-dimer [1], is subject to three unique post-translational modifications, which seem to be based on ATP-dependent formation of peptide bonds. Tubulin tyrosine ligase, which has been purified and cloned from mammalian brain, restores the C-terminal tyrosine previously removed by a carboxypeptidase-like activity from some α-tubulins [2]. Polyglutamyl and polyglycyl side chains of various lengths can be formed at the γ-carboxylate of one or more specific glutamate residue(s) via an isopeptide bond defining the branch site [3,4]. Polyglycylation is found in many [4–6] but not all axonemes [7,8]. Polyglutamylation, originally discovered by mass spectrometry of mammalian brain tubulin peptides [3], is a general tubulin modification, which extends to the oldest eukaryotes [7–9] but, as shown here, seems to be absent in yeast.

Two enriched enzyme preparations of tubulin polyglutamylase are currently available. One is derived from the brain of 3 day old mice [10], the other is obtained by stripping the stable microtubular cytoskeleton of the trypanosomatid *Crithidia fasciculata* with moderate salt and by subsequent purification steps [11]. Similarities and differences of the two enzyme preparations have been discussed. In addition, we noted that two synthetic peptides with an oligoglutamyl side chain, corresponding to the carboxy-terminal end of brain α- and β-tubulin, are accepted by the *Crithidia* enzyme, albeit at a low efficiency [11]. Using a broader collection of tubulin-related peptides, we now show that the presence of a free α-carboxylate on a glutamic acid residue is necessary and sufficient for peptide glutamylation catalysed by *Crithidia* tubulin polyglutamylase. Since the product of each glutamylation reaction fulfills the substrate requirement for the next cycle, a progressive elongation of side chains is achieved. With brain tubulin as a substrate, analysis of the terminal peptides identified variants carrying up to 50 glutamic acid residues.

2. Materials and methods

C. fasciculata culture, the purification of tubulin polyglutamylase from the 0.25 M salt extract of isolated cytoskeletons and the glutamylation assays were as described [11]. Synthetic peptides were incubated at 0.1 mM with partially purified enzyme (fraction II after ATP affinity chromatography), 4 mM glutamic acid and 2 mM ATP in the standard assay.

Glutamylation products were analysed by reverse-phase high performance liquid chromatography (HPLC), mass spectrometry and automated Edman degradation as described [11]. Alternatively, a 10 μl aliquot of the reaction mixture was diluted to 400 μl with 20 mM Tris-HCl, pH 8 (buffer A), and subjected to anion exchange chromatography on a TSKgel DEAE-NPR column (Tosohaas, Stuttgart, Germany). The column dimensions were 4.6×35 mm. Peptides were eluted with a 2.7 ml linear gradient of 0–0.5 M NaCl in buffer A followed by a 0.9 M salt wash. The flow rate was 200 μl/min and 100 μl fractions were collected. Peptides present in the DEAE fractions were processed for mass spectrometry using C18-ZipTips (Millipore, Bedford, MA, USA) according to the instructions of the manufacturer.

Purified tubulin from *Saccharomyces cerevisiae* was purchased from Cytoskeleton (Denver, CO, USA). An additional preparation was kindly provided by Dr. E. Schiebel (Glasgow, UK). Yeast tubulin was glutamylated at 0.1 mg/ml, pig brain tubulin at 0.3 mg/ml, with 4 mM [^{3}H]Glu and 0.1 μg of a partially purified polyglutamylase fraction obtained after ATP affinity chromatography. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in the presence of 6 M urea to separate α- and β-tubulins. Immunoblots were analysed with monoclonal antibodies YL1/2, specific for tyrosinated α-tubulin [12], the β-tubulin-specific antibody KMX-1 [13] and the glutamylation-specific antibody GT335 [14]. ^{3}H-autoradiography was performed using BioMax TranScreen LE (Kodak, Rochester, NY, USA).

3. Results

3.1. Polyglutamylation of synthetic tubulin peptides

We previously found [11] that synthetic peptides corresponding to the carboxy-terminal 19 and 15 residues of β- and α-brain tubulin are substrates of *Crithidia* tubulin polyglutamylase, provided that they carried an oligoglutamyl side chain of four residues on the glutamate at position 9, i.e. the established glutamylation sites Glu-445 for α1/α2-isoforms and Glu-435 for class II β-tubulin [3,15,16]. We now used a set of different synthetic peptides to characterise the sequence requirements for peptide glutamylation in detail. Fig. 1A summarises the results of the peptide assays.

Reduction of the length of the side chain to the monoglu-

*Corresponding author. Fax: (49) (551) 201 1578.

PII: S0014-5793(99)01227-2

A

peptide	sequence	substrate	ΔE
α 0E	VDSVEGEGEEEGEEY	–	0
α 0E (-Y)	VDSVEGEGEEEGEE	+	1-3
α 0E (Δ2)	VDSVEGEGEEEGE	+	1-3
α 0E (Δ3)	VDSVEGEGEEEG	–	0
α 1E	VDSVEGEG**E**EEGEEY (1)	++	1-6
α 4E	VDSVEGEG**E**EEGEEY (4)	++	1-6
α 1E (+2)	VDSVEGEGEE**E**GEEY (1)	++	1-6
α 1E (-2)	VDSVEG**E**GEEEGEEY (1)	++	1-6
β 0E	DATADEQGEFEEEEGEDEA	–	0
β 0E (-A)	DATADEQGEFEEEEGEDE	+	1-4
β 0E (-EA)	DATADEQGEFEEEEGED	–	0
β 1E	DATADEQG**E**FEEEEGEDEA (1)	++	1-5
β 4E	DATADEQG**E**FEEEEGEDEA (4)	++	1-6
β 1E (-3)	ATAD**E**QGEFEEEEGEDEA (1)	+	1-3
β 1E (+9)	ATADEQGEFEEEEGED**E**A (1)	+	1-3

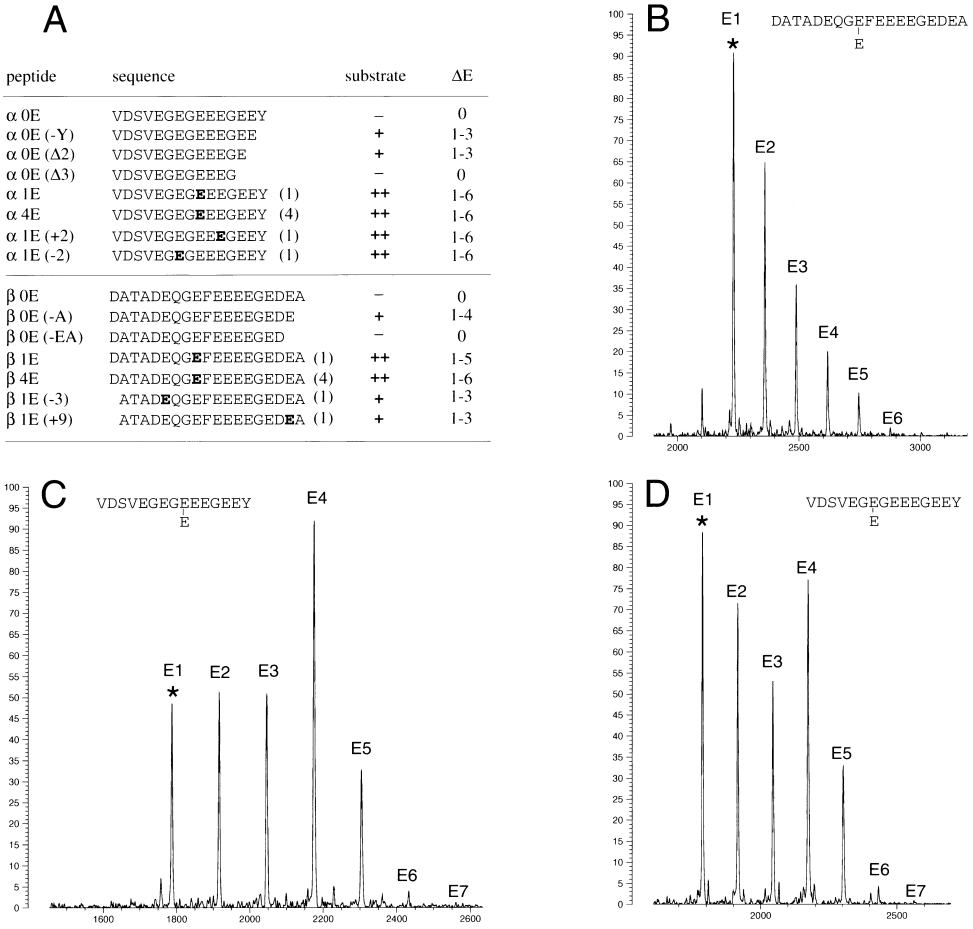

Fig. 1. Substrate-specificity of *Crithidia* tubulin polyglutamylase in a peptide system. A: Summary of the results with the peptide assays. Bold letters in the sequences indicate the positions of the oligoglutamyl side chains. The number in brackets past the sequence gives the length of the glutamyl side chain of the substrate. Substrate activity is given in the penultimate column. The last column (ΔE) gives the number of glutamyl residues incorporated. B: Mass spectrum of the reaction product obtained with the normal monoglutamylated β-peptide. The sequence of the substrate is given at the top. A star marks the corresponding peak in the mass spectrum. The additional peaks are numbered according to the length of the glutamyl side chain. Up to five additional glutamyl residues are incorporated. C: Mass spectrum of the reaction product obtained with the normal monoglutamylated α-peptide showing the incorporation of up to six additional glutamyl residues. D: Elongation of a monoglutamylated α-peptide with the side chain shifted by two positions (see sequence at the top). Up to six additional residues are incorporated.

tamylated derivatives had no influence on the glutamylation reaction. Up to five and six additional glutamates were incorporated into the branched α- (Fig. 1C) and β-peptide (Fig. 1B). Automated Edman degradation and carboxypeptidase A treatment confirmed that the additional glutamyl residues were added to the existing side chain of the substrate. Next, we tested a series of peptides in which the monoglutamyl side chain was attached to different glutamate residues of the main chain. Shifts in α were by plus or minus two residues while for β, shifts of minus two and plus nine residues were tried. These derivatives were all accepted as substrates (Fig. 1A,D). All peptides recognised so far as substrates have a single glutamate with a free α-carboxylate group, provided by the side chain branch, since the carboxy-terminal residue is either a tyrosine (α) or an alanine (β). We therefore tested a series of carboxy-terminally truncated α- and β-peptides without a side chain. All peptides ending with glutamate were recognised as substrates and automated sequencing confirmed that the extra glutamyl residues incorporated (Fig. 1A) were added to the carboxy-terminal end. For instance, the peptides corresponding to detyrosinated α-tubulin or to α-tubulin lacking an additional glutamate (Δ2 tubulin) increased by up to three glutamyl residues. Judged by the maximal number of glutamyl units added and by the relative peak heights observed in mass spectrometry, the elongation at the carboxy-terminal end was somewhat less efficient than the elongation of the side chains in the other peptides. In contrast, peptides ending with glycine or aspartic acid as well as the full length peptides ending with tyrosine or alanine were not accepted by the enzyme (Fig. 1A).

Anion exchange chromatography on a DEAE column provided an additional and convenient assay of the glutamylation reaction (Fig. 2). Peptides were resolved according to the length of their side chains and the absorption profile directly visualised the glutamylation process. However, the least abundant species detected by mass spectrometry (Fig. 1A,C) were not visible in the absorption profile (Fig. 2). Even after 20 h, the original monoglutamylated substrate peptides were still the major components in the reaction mixture, indicating the rather low efficiency of peptide glutamylation.

3.2. Hyperglutamylation of brain tubulin

We also analysed the glutamylation of mammalian brain tubulin by tubulin polyglutamylase [11] in more detail. Radioactive glutamic acid was incorporated up to levels of 70 mol per mol αβ-tubulin heterodimer (Fig. 3A). Analysis of aliquots of the reaction taken at 0 and 16 h showed in SDS-PAGE a striking reduction of the electrophoretic mobility of both α- and β-tubulin upon polyglutamylation. The stained tubulin bands were electro-eluted from the gel, digested with endoproteinase Lys-C (α-tubulin) and CNBr (β-tubulin), respectively, and the acidic carboxy-terminal peptides were purified by anion exchange chromatography and reverse-phase HPLC [7–9]. Mass spectrometric analysis of the various HPLC fractions revealed extensive polyglutamylation. Up to 31 additional glutamyl residues were found for the α-peptides. Fig. 3C shows a mass spectrum for a fraction containing detyrosinated α-peptides with 8–27 extra glutamyl residues. Automated sequencing revealed only one side chain situated as expected at Glu-445. The highly glutamylated α-peptides were found in the tyrosinated and detyrosinated form and in a subset additionally modified by phosphorylation at Ser-439 as noted previously for a synthetic α-peptide [11]. While the most abundant variants carried 15–17 extra glutamyl residues, we also observed a minor fraction of the unmodified peptide and of peptides with shorter side chains. In contrast, tubulin purified from brain usually contains up to four or six extra glutamyl residues per α- and β-tubulin [3,15,16]. We also observed the peptides from the α4-isoform with up to 32 glutamyl residues. Currently, we do not know how they distribute over the two side chains located at glutamic acid residues 443 and 445 [11,17].

In a different experiment, the carboxy-terminal peptides were directly generated from the glutamylation reaction without the preparative SDS-PAGE step. Under these conditions, even higher glutamylation levels were recorded by mass spectrometry. Both α- and β-peptides contained up to 50 extra glutamyl residues with the average number centring around 35 (data not shown). This value is in good agreement with the molar level calculated from the radioactive glutamate incorporation (Fig. 3A).

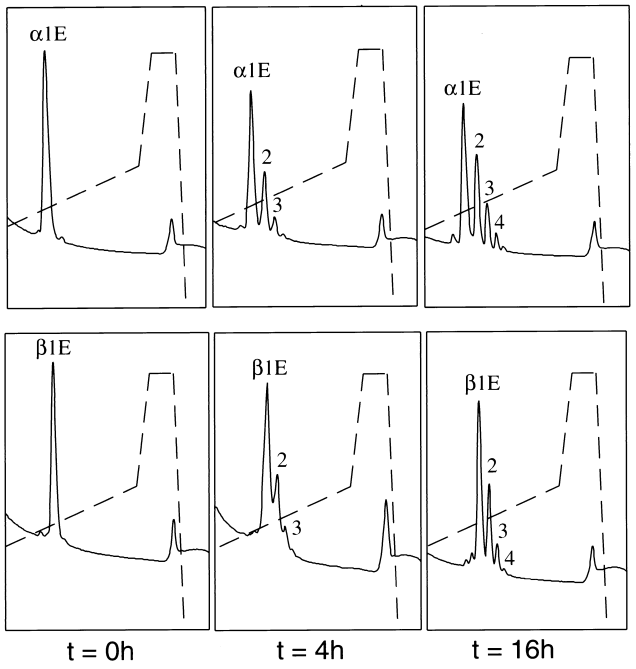

Fig. 2. Glutamylation of synthetic peptides by *Crithidia* polyglutamylase, monitored by anion exchange chromatography on a DEAE column. Aliquots of the reaction with the monoglutamylated α-peptide (α1E, upper panel) and the normal monoglutamylated β-peptide (β1E, lower panel) were taken at the indicated times. The reaction was followed by absorption at 214 nm. Peaks are numbered according to the length of the glutamyl side chain obtained by mass spectrometry. For peptide sequences, see Fig. 1A.

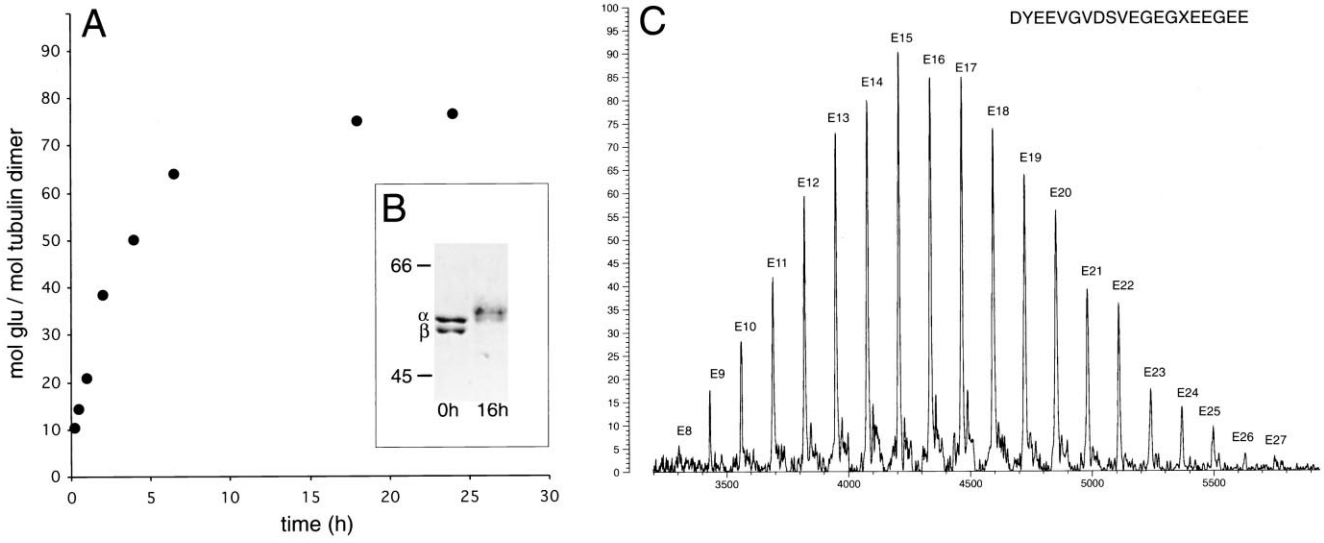

Fig. 3. Extensive glutamylation of mammalian brain tubulin catalysed by *Crithidia* tubulin polyglutamylase. A: Time-course of tubulin glutamylation followed by incorporation of radioactive glutamic acid and expressed as the number of glutamyl residues added per tubulin dimer. B: SDS-PAGE of regular and highly glutamylated brain tubulin, obtained by a 16 h treatment with the *Crithidia* enzyme. Note the lower mobility of the α- and β-tubulin bands. C: Mass spectrum of the C-terminal peptides of glutamylated α-tubulin, obtained by digestion with endoproteinase Lys-C. The result of automated sequence analysis of the corresponding fraction is given at the top. The variants have side chain lengths between eight and 27 glutamyl residues. They are derived from detyrosinated α-tubulin and also show complete phosphorylation of the single serine residue. The X in the sequence marks the position of the glutamyl side chain, i.e. Glu-445.

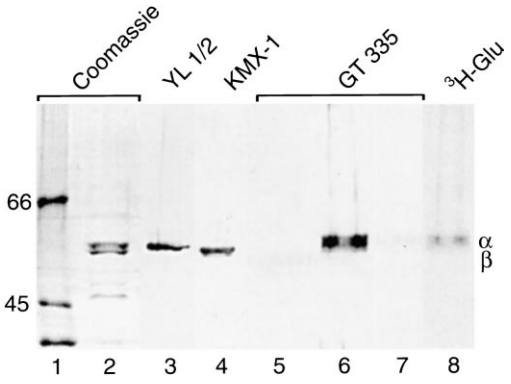

Fig. 4. Immunological evidence for a monoglutamylase activity. Yeast tubulin was separated by SDS-PAGE (lane 2, marker proteins in lane 1, molecular weights given in kDa) and α- and β-bands are stained in immunoblots (lanes 3 and 4) by monoclonal antibodies YL1/2 (α) and KMX-1 (β). The glutamylation-specific antibody GT335 does not recognise yeast tubulin (lane 5) unless it is treated with a partially purified glutamylase fraction from *Crithidia* (lane 6). Control blot of enzyme processed without yeast tubulin through the standard assay (lane 7). A parallel [^{3}H]Glu autoradiography demonstrates the incorporation of glutamic acid into yeast α-tubulin (lane 8).

3.3. Immunological evidence for a monoglutamylation activity

Since none of the synthetic peptides can be used to monitor the formation of the isopeptide bond involved in the synthesis of the first glutamyl side chain residue, we searched for a different detection system. Fig. 4 gives the results of SDS-PAGE and immunoblots on purified yeast tubulin. The barely resolved α- and β-tubulins are recognised by the monoclonal antibodies YL1/2 and KMX-1, which are specific for α- and β-tubulin, respectively. In contrast, no reaction was observed with the antibody GT335, which recognises a motif already provided by monoglutamylation of α-tubulin [14]. This situation changed when yeast tubulin previously treated with partially purified polyglutamylase was used. GT335 now detected the α-tubulin band (Fig. 4, lane 6) while a control blot of the enzyme processed without yeast tubulin through the standard assay was negative with GT335 (Fig. 4, lane 7). A parallel blot and autoradiograph on yeast tubulin treated with [^{3}H]glutamate and enzyme confirmed that the gain of GT335 reactivity was due to the incorporation of glutamic acid into α-tubulin (Fig. 4, lane 8). The combined results indicate that purified yeast tubulin is not glutamylated and that an enzyme activity present in our preparation can introduce the first glutamic acid of a side chain via isopeptide bond formation.

4. Discussion

We used a set of synthetic peptides related to the carboxy-terminal end of brain α- and β-tubulin to characterise the substrate-specificity of a partially purified preparation of tubulin polyglutamylase from the trypanosomatid *C. fasciculata* [11]. The combined results (Fig. 1A) show that peptides were subject to polyglutamylation if they displayed a free α-carboxylate group of a glutamic acid residue. This minimal sequence requirement was already fulfilled by a series of monoglutamylated peptides carrying a single glutamate connected by an isopeptide bond to a glutamate of the main peptide chain and by those unbranched peptides ending with a carboxy-terminal glutamate residue. In the peptide system, extension of an already existing side chain was favoured over the extension past a carboxy-terminal glutamate. The specificity for a free α-carboxylate provided by a glutamic acid residue was absolute, internal glutamic acid residues were not modified and terminal residues other than glutamic acid were not recognised. Within this peptide system, no isopeptide bond formation ('branching') was observed. The length and relative position of an existing side chain had no obvious influence on the elongation. Thus, the polyglutamylase has a low sequence requirement and its activity is triggered once it encounters a monoglutamylated α- or β-tubulin. This low sequence requirement can explain the generation of very long side chains when brain tubulin is used as a substrate (Fig. 3). The product of each glutamylation step again fulfills the sequence requirements necessary for the next cycle. Thus, glutamyl residues are added sequentially onto tubulin, resulting in progressive elongation of the oligoglutamyl side chain (see below).

Brain tubulin is by far a much better substrate of tubulin polyglutamylase than the various synthetic peptides. As argued before for tubulin tyrosine ligase [18], the peptides may not make optimal contacts with the modifying enzyme or may be less able to adopt the correct conformation for optimal polyglutamylation. Treatment of purified brain tubulin results in a hyperglutamylated preparation containing up to 50 extra glutamyl residues per α- and β-tubulin. These values exceed the highest values reported for in vivo glutamylated tubulins, which range from 15 for *Trypanosoma brucei* [7] and 17 for the basal apparatus of a green alga [19] to 22 for *Crithidia* α-tubulin (our unpublished results). Indeed, the high effectiveness of hyperglutamylation, so far only observed in vitro, invites the possible need for an opposing enzymatic activity acting as a deglutamylase. Some evidence for such an activity has also emerged from in vivo studies [20]. On the other hand, our results on different methods to isolate the carboxy-terminal peptides of enzymatically glutamylated brain tubulin open the possibility that the most extensively glutamylated species can be lost in some procedures [7–9,19]. Highly glutamylated tubulin species migrate aberrantly in SDS-PAGE (Fig. 3B and Fig. 9 in reference [10]) and their carboxy-terminal peptides may be recovered in a lower yield by in gel digestion and moved from the bulk part in ion exchange chromatography. Thus, previously reported values [7–9,19] may be an underestimate of in vivo polyglutamylation.

The low sequence requirement of the *Crithidia* polyglutamylase in the peptide assay contrasts the high site selectivity observed in various α- and β-tubulins. In most cases, only a particular glutamic acid residue in a short sequence, rich in glutamic acids, carries the branched side chain [3,7,15,16]. Thus, it seems that the site-specificity, which necessarily involves an isopeptide bond, is established by a monoglutamylase, which initiates the correct side chain due to complex formation with tubulin. Such a monoglutamylase together with polyglutamylase activity was observed in a partially purified enzyme preparation from 3 day old mice [10]. Using yeast tubulin, which seems to be unglutamylated, as a substrate and the monoclonal antibody GT335 as a detection system, we found a convenient assay for monoglutamylation activity (Fig. 4). This immunological assay can serve in the future for purification of the monoglutamylase.

Acknowledgements: We thank Drs. Joel Vandekerckhove and Jürgen Wehland for a number of synthetic peptides. Drs. Bernard Eddé and Klaus Ersfeld kindly provided the tubulin antibodies GT335 and KMX-1, respectively. We also acknowledge a generous gift of yeast tubulin by Dr. Elmar Schiebel.

References

[1] Nogales, E., Wolf, S.G. and Downing, K.H. (1998) Nature 391, 199–202.
[2] Ersfeld, K., Wehland, J., Plessmann, U., Dodemont, H., Gerke, V. and Weber, K. (1993) J. Cell Biol. 120, 725–732.
[3] Eddé, B., Rossier, J., Le Caer, J.-P., Desbruyères, E., Gros, F. and Denoulet, P. (1990) Science 247, 83–84.
[4] Redeker, V., Levilliers, N., Schmitter, J.-M., Le Caer, J.-P., Rossier, J., Adouette, A. and Bré, M.H. (1994) Science 266, 1688–1691.
[5] Multigner, L., Pignot-Paintrand, I., Saoudi, Y., Job, D., Plessmann, U., Rüdiger, M. and Weber, K. (1996) Biochemistry 35, 10862–10871.
[6] Mary, J., Redeker, V., Le Caer, J.-P., Rossier, J. and Schmitter, J.M. (1996) J. Biol. Chem. 271, 9928–9933.
[7] Schneider, A., Plessmann, U. and Weber, K. (1997) J. Cell Sci. 110, 431–437.
[8] Schneider, A., Plessmann, U., Felleisen, R. and Weber, K. (1998) FEBS Lett. 429, 399–402.
[9] Weber, K., Schneider, A., Westermann, S., Müller, N. and Plessmann, U. (1997) FEBS Lett. 419, 87–91.
[10] Regnard, C., Audebert, S., Desbruyères, E., Denoulet, P. and Eddé, B. (1998) Biochemistry 37, 8395–8404.
[11] Westermann, S., Schneider, A., Horn, E. and Weber, K. (1999) J. Cell Sci. 112, 2185–2193.
[12] Wehland, J., Schroeder, H.C. and Weber, K. (1984) EMBO J. 3, 1295–1300.
[13] Birkett, C.R., Foster, K.E., Johnson, L. and Gull, K. (1985) FEBS Lett. 187, 211–218.
[14] Wolff, A., de Néchaud, B., Chillet, D., Mazarguil, H., Desbruyères, E., Audebert, S., Eddé, B., Gros, F. and Denoulet, P. (1992) Eur. J. Cell Biol. 59, 425–432.
[15] Rüdiger, M., Plessmann, U., Klöppel, K., Wehland, J. and Weber, K. (1992) FEBS Lett. 308, 101–105.
[16] Redeker, V., Melki, R., Promé, D., Le Caer, J.-P. and Rossier, J. (1992) FEBS Lett. 313, 185–192.
[17] Redeker, V., Rossier, J. and Frankfurter, A. (1998) Biochemistry 37, 14838–14844.
[18] Rüdiger, M., Wehland, J. and Weber, K. (1994) Eur. J. Biochem. 220, 309–320.
[19] Geimer, S., Teltenkötter, A., Plessmann, U., Weber, K. and Lechtreck, K.-F. (1997) Cell Motil. Cytoskel. 37, 72–85.
[20] Audebert, S., Desbruyères, E., Gruszcynski, C., Koulakoff, A., Gros, F., Denoulet, P. and Eddé, B. (1993) Mol. Biol. Cell 4, 615–626.