

Isolation of tubulin polyglutamylase from *Crithidia*; binding to microtubules and tubulin, and glutamylation of mammalian brain α - and β -tubulins

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SUMMARY

Trypanosomatids have a striking cage-like arrangement of submembraneous microtubules. We previously showed that α - and β - tubulins of these stable microtubules are extensively modified by polyglutamylation. Cytoskeletal microtubular preparations obtained by Triton extraction of *Leishmania tarentolae* and *Crithidia fasciculata* retain an enzymatic activity that incorporates radioactive glutamic acid in a Mg^{2+} -ATP-dependent manner into α - and β -tubulins. The tubulin polyglutamylase is extracted by 0.25 M salt. The *Crithidia* enzyme can be purified by ATP-affinity chromatography, glycerol-gradient centrifugation and ion-exchange chromatography. After extraction from the microtubular cytoskeleton the glutamylase forms a complex with $\alpha\beta$ tubulin, but behaves after removal of tubulin as a globular protein with a molecular mass of 38×10^3 . In highly

enriched fractions a corresponding band is the major polypeptide visible in SDS-PAGE. The enzyme from *Crithidia* recognises mammalian brain tubulin, where it incorporates glutamic acid preferentially into the more acidic variants of both α - and β -tubulins. Synthetic peptides with an oligoglutamyl side chain, corresponding to the carboxy-terminal end of brain α - and β -tubulins, are accepted by the enzyme, albeit at low efficiency. The polyglutamylase elongates the side chain by up to 3 and 5 residues, respectively. Other properties of the tubulin polyglutamylase are also discussed.

Key words: Microtubule, Post-translational modification, Polyglutamylation, Trypanosomatid, Tubulin

INTRODUCTION

Microtubules are necessary for cell division, directed intracellular transport, the beating of cilia and flagella and the dynamic organisation of cell morphology. The structural unit of microtubules is the $\alpha\beta$ heterodimer, where α - and β -tubulins are usually encoded by multigene families. Additional tubulin heterogeneity arises from a series of post-translational modifications (PTMs), which fall into two categories. Some, such as acetylation and phosphorylation, are more general PTMs while others seem tubulin-specific and always involve the acidic carboxy-terminal region, which shows remarkable sequence variation in different tubulins. The terminal tyrosine of certain α -tubulins participates in a tyrosination cycle. This is based on a carboxypeptidase-like activity and on the tubulin tyrosine ligase, which restores the tyrosine in an ATP-dependent manner. Additional loss of the penultimate glutamate provides a tubulin derivative ($\Delta 2$ -tubulin) that is no longer a substrate for the ligase. Moreover, polyglutamyl and polyglycyl side chains of various lengths can be added via an isopeptide bond to the γ -carboxylate of a specific glutamate residue(s) of α - and β -tubulins (for reviews, see Luduena, 1998; MacRae, 1997). The tubulin-specific PTMs involve the carboxy-terminal region, which is not visible in the atomic

model of the $\alpha\beta$ dimer (Nogales et al., 1998). Of the various enzymes involved in tubulin-specific PTMs, so far only the tubulin tyrosine ligase from brain tissue has been obtained in pure form (Schröder et al., 1985). Its sequence (Ersfeld et al., 1993) seems to share a fold with the glutathione synthetase ADP-forming family (Dideberg and Bertrand, 1998).

Polyglutamylation, originally discovered by mass spectrometry of the carboxy-terminal peptides of mammalian brain tubulin (Eddé et al., 1990) is of particular interest, since it seems to be one of the oldest PTMs of tubulin. It is documented by mass spectrometry for *Trypanosoma brucei*, a member of the *Kinetoplastida*, for *Trichomonads* and for the diplomonad *Giardia lamblia* (Schneider et al., 1997, 1998; Weber et al., 1997). In contrast, among these early eukaryotes the tyrosine cycle is restricted to trypanosomes (Schneider et al., 1987, 1997; Sherwin et al., 1987). Work on primary neuronal cultures showed that polyglutamylation is reversible in vivo and microtubules seem a better substrate than the heterodimeric tubulin (Audebert et al., 1993). In agreement with this, centrioles of cultured mammalian cells are glutamylated, as judged by immunofluorescence microscopy with a specific antibody (Bobiniec et al., 1998), and an α -tubulin population of basal apparatus, the homologue structure for centrioles in green algae, contains up

to 17 glutamic acid residues in the side chain (Geimer et al., 1997). In vitro results raise the possibility that polyglutamylation regulates the interaction between tubulin and structural microtubule-associated proteins as well as between tubulin and motor proteins (Boucher et al., 1994; Larcher et al., 1996).

Searching for a source from which to isolate a tubulin polyglutamylase we have concentrated for several reasons on trypanosomatids. First, some of them, like *Leishmania tarentolae* and *Crithidia fasciculata*, can be grown on a large scale in rather inexpensive medium. Second, in these simple protozoa one expects a lower protein complexity than in mammalian brain. Third, we have previously shown for *Trypanosoma brucei* that the stable microtubules of the subpellicular cage-like cytoskeleton (Seebeck et al., 1988, 1990) are extensively glutamylated (Schneider et al., 1997). Nonglutamylated tubulins are only present in trace amounts and polyglutamyl side chains range up to 15 and 6 residues for α - and β -tubulin respectively. Fourth, *L. tarentolae* offers at a later stage a functional analysis of the glutamylase activity by gene replacement experiments (Cruz et al., 1991; ten Asbroek et al., 1990).

Here we show that the microtubular cytoskeleton of *Leishmania* and *Crithidia* obtained by Triton extraction retains the polyglutamylase, which can be stripped by moderate salt concentrations. We describe the purification of the *Crithidia* enzyme by ATP-affinity chromatography, glycerol-gradient centrifugation and anion-exchange chromatography. The enzyme behaves as a globular protein of molecular mass 38×10^3 and recognises mammalian brain tubulin as a substrate.

MATERIALS AND METHODS

Cells

Crithidia fasciculata were cultured at 26°C in defined medium (Shim and Fairlamb, 1988) with 4 µg/ml hemine. *Leishmania tarentolae* (UC strain) was grown at 27°C in Difco brain heart infusion medium containing 10 µg/ml hemine. Cells were grown in 1 l flasks with gentle shaking. Cell densities were determined with a hemacytometer.

Assays for tubulin polyglutamylase

Enzyme activity was assayed by the incorporation of [³H]glutamic acid into tubulin. The 50 µl standard reaction mixture contained 50 mM Tris-HCl (pH 9.0 or 7.0), 15 mM KCl, 5 mM MgCl₂, 0.5% NP-40, 1 mM ATP (adjusted to pH 7.0 with NaOH), 5 mM DTT, 20% glycerol and L-[³H]glutamic acid (60 Ci/mmol, Biotrend, Cologne, Germany) diluted with unlabeled L-glutamic acid at various concentrations.

To demonstrate glutamylation activity of isolated cytoskeletons during the course of the initial studies, 20–40 µg of detergent-extracted cytoskeletons were resuspended in the assay mixture and incubated at 28°C, usually for 90 minutes. The reaction was stopped by the addition of SDS-sample buffer and subsequent boiling for 3 minutes. Polypeptides were separated by SDS-PAGE (7.5% gel) and electroblotted onto nitrocellulose. After staining with Ponceau S the prominent tubulin bands were cut out, destained in water, dissolved in 5 ml FilterCount (Packard, Groningen, The Netherlands) and subjected to liquid scintillation counting. During purification and characterisation of tubulin polyglutamylase a rapid and more convenient assay was used. Microtubule protein from porcine brain was used as substrate at a final concentration of 0.4 mg/ml in the assay. After incubation with enzyme (20 µg crude solubilised enzyme or approximately 10 ng enzyme after anion-exchange chromatography) the reaction mixture was transferred onto a paper disc (Whatman

3MM, 2.3 cm diameter), placed in ice-cold 10% (w/v) trichloroacetic acid (TCA) and washed as described (Flavin and Murofushi, 1984) to remove unbound radioactivity. After drying the filter discs were subjected to scintillation counting with 5 ml FilterCount. Background values (100–200 cpm) were subtracted.

Preparation of cytoskeletons and solubilization of tubulin polyglutamylase

All steps were carried out at 4°C. Starting with the extraction step the following protease inhibitors were included in all buffers: 100 µM Pefabloc SC, 5 µg/ml leupeptin, 1 µg/ml pepstatin and 10 µg/ml aprotinin (Biomol, Hamburg, Germany).

Cells were harvested from a 2 l culture during late log phase ($3\text{--}4 \times 10^7$ cells/ml) by centrifugation at 3000 g for 5 minutes and washed twice in cold phosphate-buffered saline. Trypanosomatids were resuspended at a concentration of approx. 1×10^9 cells/ml in hypotonic extraction buffer, containing 10 mM Piperazine-N,N'-bis(2-ethane sulfonic acid) (Pipes-KOH), pH 6.9, 5 mM EDTA, 1 mM DTT, 1% Triton X-100, and left on ice for 5 minutes before extraction using a closely fitting Dounce homogenizer. After centrifugation at 20000 g for 10 minutes, this step was repeated. Pelleted cytoskeletons were washed in buffer A (100 mM Pipes, pH 6.9, 1 mM MgCl₂, 1 mM EGTA, 1 mM DTT) and centrifuged as above. Solubilisation of tubulin polyglutamylase was achieved by homogenisation of the washed cytoskeletons in 10 ml buffer A containing 0.25 M NaCl. After centrifugation (Beckman TL100.3, 40000 rpm, 20 minutes) the solubilisation step was repeated. The supernatants were combined and clarified by centrifugation (TL100.3, 55000 rpm, 10 minutes) to yield a crude fraction of solubilised tubulin polyglutamylase (Fraction I). The crude enzyme can be frozen in liquid nitrogen and kept at –80°C. The addition of 20% glycerol improved the enzyme stability.

Purification of tubulin polyglutamylase

Step 1: ATP-affinity chromatography

Periodate-oxidized ATP was coupled to CNBr-activated Sepharose 4B (Amersham Pharmacia, Uppsala, Sweden) via a spacer of sebacic acid dihydrazide as described (Flavin and Murofushi, 1984). Fraction I was applied to a 1 ml affinity column equilibrated in buffer B (20 mM Pipes, pH 6.9, 5 mM MgCl₂, 1 mM DTT) by gravity flow at a rate of 1 ml/minute. The column was washed with 10 column volumes of buffer B+0.25 M KCl. The glutamylation activity was eluted with buffer B containing 20 mM ATP, 20 mM MgCl₂ and 0.5 M KCl. 1 ml fractions were collected and during this elution the flow rate was reduced to 0.5 ml/minute. Fractions containing glutamylase activity (Fraction II) were concentrated using Centricon 10 devices (Amicon, Witten, Germany). To improve enzyme recovery, the devices were pretreated with 5% (v/v) Tween 20 according to the manufacturer's instructions.

Step 2: glycerol-gradient centrifugation

Fraction II enzyme was subjected to glycerol-gradient centrifugation. 300 µl of concentrated fraction II was loaded on to each linear 10%–20% (v/v) glycerol-gradient in 20 mM Pipes, pH 6.9, 1 mM MgCl₂, 1 mM EGTA, 1 mM DTT (buffer C). Samples were centrifuged at 4°C for 20 hours at 286000 g in an SW 41-rotor (Beckman). Tubes were punctured and 30 fractions of 0.4 ml each were collected from each gradient. Tubulin polyglutamylase-positive fractions were pooled to obtain fraction III. For the estimation of sedimentation coefficients, marker proteins (ribonuclease A, ovalbumin, BSA and muscle aldolase) were run in parallel tubes. A spot assay for tubulin was performed by transferring 1 µl of each gradient fraction onto nitrocellulose. After saturating the membrane with BSA, tubulin was detected using the monoclonal α -tubulin antibody DM1 A (Sigma, Deisenhofen, Germany) and ECL-detection (Amersham).

Step 3: anion-exchange chromatography

A portion of fraction III enzyme was further purified by anion-exchange chromatography. 2 ml of fraction III were applied to a Mono

Q PC1.6/5 column (SMART system, Pharmacia, Uppsala, Sweden). After sample loading the column was eluted with a 4.0 ml linear gradient generated by mixing buffer C with buffer D (buffer C+1.0 M NaCl) in the following proportions: 0-2.3 ml, 0% D; 2.3-3.3 ml, 0-50% D; 3.3-3.5 ml, 50-90% D; 3.5-4.0 ml, 90% D. The flow rate was 100 μ l/minute and 100 μ l fractions were collected (fraction IV).

Fraction IV enzyme is very dilute. It can be stored at -80°C after addition of 2 mg/ml BSA and 20% glycerol. Fraction II can be stored in the presence of 20% glycerol at -80°C . Fraction III may be stored overnight at 4°C or at -80°C for longer periods of time.

Protein concentrations were determined according to Bradford (1976) using the Bio-Rad (Munich, Germany) reagent and BSA as a standard. For very dilute samples a protein quantification kit (Chemicon Inc. Tenecula, CA, USA) was used according to the instructions of the manufacturer (sensitivity, approximately 1 ng BSA).

2D-gel electrophoresis and autoradiography

For analysis of tubulin glutamylation products, typically 50 μ g of brain microtubule protein were glutamylated using enzyme fractions in the standard assay. Proteins were precipitated with chloroform-methanol (Wessel and Fluegge, 1984) and dissolved in 50 μ l isoelectric focussing (IEF)-lysis buffer (O'Farrell, 1975). In the first dimension, high resolution IEF was performed in cylindrical gels (3 \times 200 mm) (Wolff et al., 1992) using pH 5-6 ampholytes (Serva, Heidelberg, Germany). The second dimension was SDS-PAGE (8% acrylamide, 0.11% bisacrylamide) on 12 cm long slab gels. For ^3H -fluorography gels were treated with Amplify (Amersham) according to the manufacturer, dried on Whatman 3MM and exposed to pre-flashed Hyperfilm MP (Amersham) at -80°C typically for 48 hours.

Glutamylation of synthetic peptides

Peptides were synthesised and purified by standard procedures. They were characterised by automated sequencing and mass spectrometry using a KRATOS MALDI 4 time of flight mass spectrometer (Shimadzu, Duisburg, Germany). Exact peptide concentrations were obtained by quantitative amino acid analysis. Peptides were incubated at a concentration of 0.1 mM with 0.1 μ g partially purified polyglutamylase (fraction II) and 4 mM glutamic acid for 16 hours using the standard assay at pH 9.0. A sample of the reaction mixture was subjected to reverse-phase HPLC. Peptide peaks were characterised by mass spectrometry and automated Edman degradation. The lack of a phenylthiohydantoin derivative at step 9 of both branched peptides was due to the four-residue oligoglutamyl side chain in line with the original synthesis (Schneider et al., 1997, 1998).

Except for the isopeptide bond at the branching point only α peptide bonds occurred in the side chain.

Miscellaneous procedures

Prior to SDS-PAGE of very dilute samples (especially of fractions from anion-exchange chromatography) proteins were precipitated using the deoxycholate-TCA method (Bensadoun and Weinstein, 1976). Polypeptide gels were visualized either by staining with Coomassie Brilliant Blue or by a silver staining procedure (Bio-Rad).

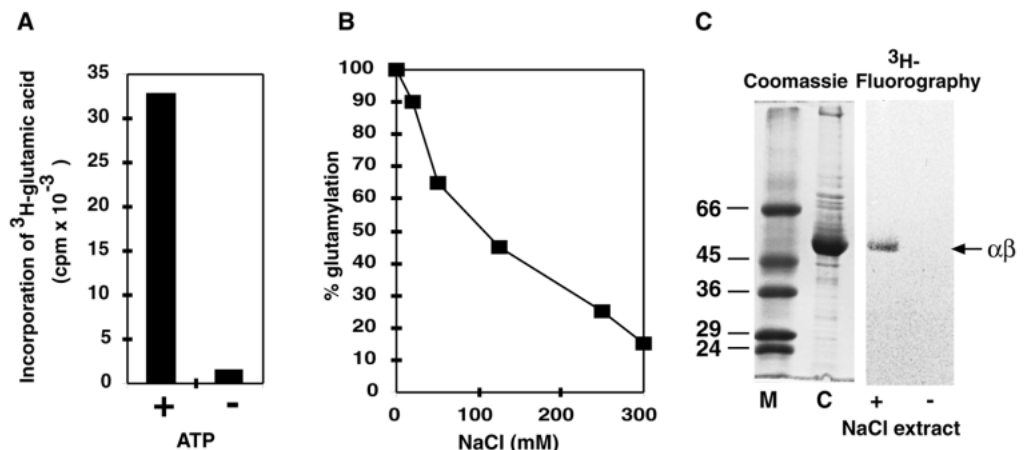
Microtubule protein from adult porcine brain was purified and associated proteins were removed by phosphocellulose chromatography (Sloboda and Rosenbaum, 1982). HeLa cell tubulin was purchased from Cytoskeleton (Denver, CO, USA). Carboxy-terminal peptides of *Leishmania* α - and β -tubulin, previously glutamylated with [^3H]glutamic acid by the crude enzyme fraction, were generated by endoproteinase LysC and CNBr digests, respectively, and purified by anion-exchange chromatography as described (Schneider et al., 1997). Peptides present in the Mono Q fractions were collected by adsorption on reverse-phase beads (Poros 50 R2, Boehringer, Mannheim, Germany) as described (Geveart et al., 1997). The beads were resuspended in a minimal amount of water and submitted to liquid scintillation counting with 5 ml Betamax (ICN, Eschwege, Germany).

RESULTS

Detergent-extracted cytoskeletons retain a tubulin polyglutamylase

Previous *in vivo* labelling experiments on *Trypanosoma brucei* cells demonstrated the tubulin-specific incorporation of glutamic acid under conditions of strict inhibition of protein biosynthesis (Schneider et al., 1997). Using an *in vitro* glutamylation assay and Triton-extracted cytoskeletons from two other trypanosomatids, *Leishmania tarantolae* and *Crithidia fasciculata*, we found that isolated cytoskeletons incorporate [^3H]glutamic acid in an ATP-dependent manner (Fig. 1A). This glutamylation activity was not found in the cytosolic fraction (data not shown). Glutamylation activity was released from the cytoskeletons by salt extraction (Fig. 1B). Tubulin-specific incorporation of glutamate was recovered by combining stripped cytoskeletons, devoid of

Fig. 1. Glutamylation activity of Triton-extracted cytoskeletons from *Leishmania tarantolae* and solubilisation of tubulin polyglutamylase. (A) Triton-extracted cytoskeletons display ATP-dependent glutamylation activity. 40 μ g *Leishmania* cytoskeletons were analysed in the standard assay described in Materials and methods. (B) Removal of glutamylation activity from isolated cytoskeletons by salt extraction. Cytoskeletons were washed in solubilisation buffer containing the NaCl concentrations indicated. The residual glutamylation activity of the cytoskeletal fraction was measured as above. (C) Incubation of salt-stripped cytoskeletons with the diluted 0.25 M NaCl extract (+NaCl extract) restores tubulin glutamylation activity. 20 μ g stripped *Leishmania* cytoskeletons (lane C) were incubated with 10 μ g 0.25 M NaCl extract; samples were separated by 10% SDS-PAGE and analyzed by ^3H -fluorography. Note that the label is incorporated solely into tubulin. Molecular masses of standard proteins (lane M) are shown.



glutamylase activity, with diluted 0.25 M NaCl extract, indicating that the NaCl extract did indeed contain the solubilized glutamylase enzyme(s). ^3H -fluorography confirmed that the label was incorporated solely into $\alpha\beta$ tubulin (Fig. 1C). When in vitro-glutamylated [^3H]Glu-tubulin was fragmented with endoproteinase LysC or with CNBr, the radioactivity eluted late from a Mono Q column. These elution profiles (not shown) correspond to the profiles of the highly acidic carboxy-terminal fragments of α - and β -tubulin (Schneider et al., 1997). Experiments with the crude soluble enzyme showed a strong inhibition of the glutamylase reaction by salt (NaCl and KCl above 100 mM). Incorporation of glutamic acid in the absence of stripped cytoskeletons serving as a substrate was low but clearly measurable. This effect is due to the presence of some tubulin in the crude soluble enzyme (see below).

Purification of tubulin polyglutamylase from *Crithidia*

The 0.25 M salt extract from *Crithidia* cytoskeletons (fraction I) was used as starting material for further purification of the tubulin polyglutamylase. Since the enzyme glutamylates homologous tubulin as well as mammalian brain tubulin (see below), microtubule protein from porcine brain, which lacks glutamylase activity, was used as a substrate throughout the purification. A filter assay measuring the incorporation of [^3H]glutamic acid into TCA-precipitable protein was used for rapid analysis of enzymatic activity.

Crude solubilised enzyme (fraction I) was subjected to ATP-affinity chromatography (Fig. 2A). The use of ATP coupled to Sepharose via a spacer of sebacic acid dihydrazide was essential, since commercial adipic acid-ATP or ATP-agarose showed a significantly lower affinity for tubulin

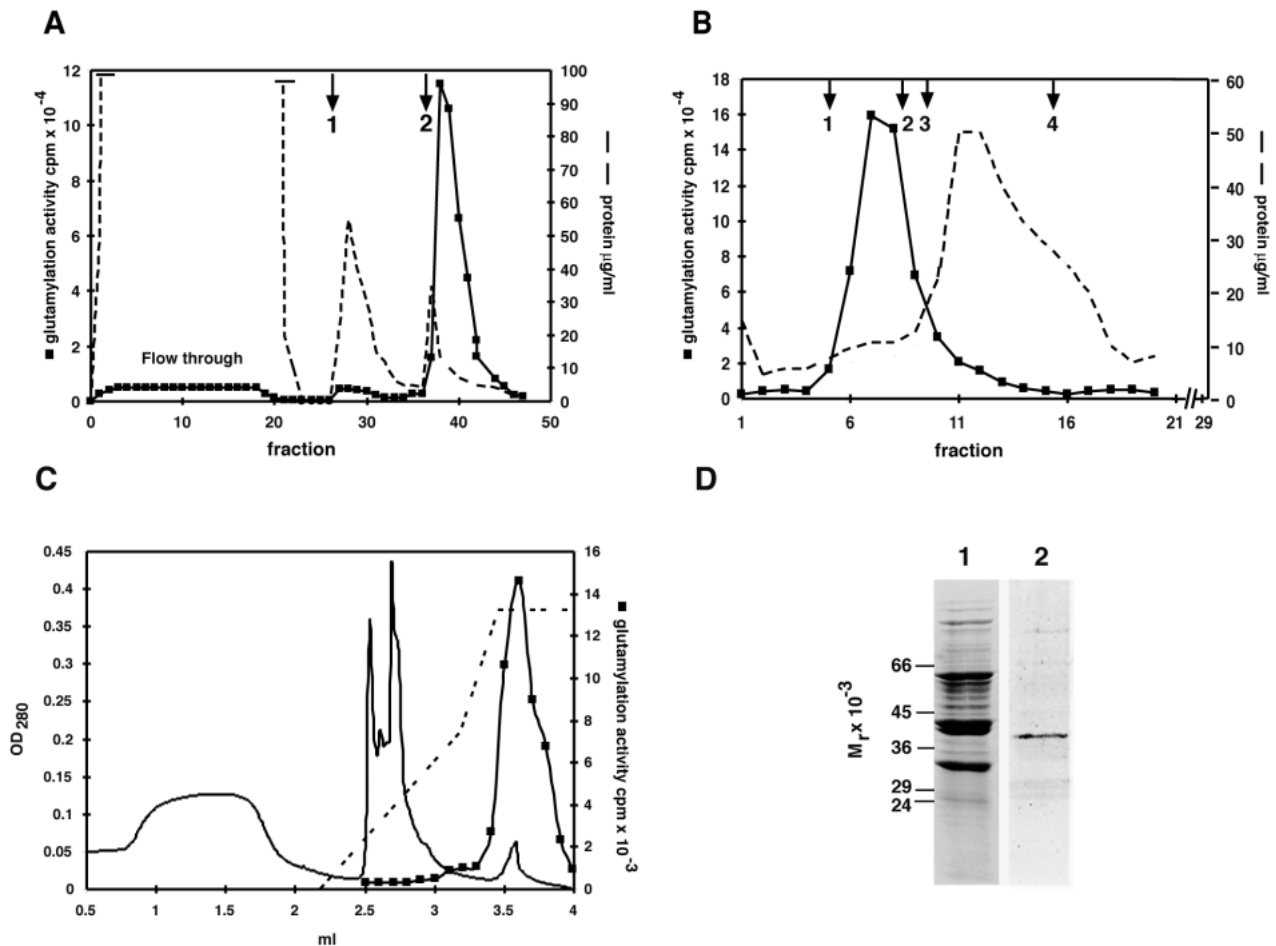


Fig. 2. Purification of tubulin polyglutamylase from *Crithidia fasciculata*. (A) Affinity chromatography of the 0.25 M salt wash of cytoskeletons (fraction I) on Sepharose-sebacic acid-ATP. At point (1) the column was washed with 0.25 M KCl; at point (2) the enzyme was eluted with 20 mM ATP, 0.5 M KCl. 1 ml fractions were collected and a 2 μl sample of each fraction was assayed for glutamylase activity (fraction II). (B) Further purification of the enzyme by glycerol-gradient centrifugation. 30 fractions were collected from each tube and 10 μl samples were assayed for glutamylase activity (fraction III). The direction of sedimentation is from left to right. The positions of marker proteins are indicated at the top (1: ribonuclease A, 2 S; 2: ovalbumin, 3.6 S; 3: BSA, 4.2 S; 4: aldolase, 7.2 S). (C) Anion-exchange chromatography of the enzyme (fraction III) on a Mono Q column. 2 μl samples were assayed for glutamylase activity (fraction IV). No enzymatic activity was detected in the combined flow-through fractions. The NaCl gradient from 0-0.9 M is indicated as a dashed line. (D) 10% SDS-PAGE of *Crithidia* tubulin polyglutamylase. Lane 1, 15 μg of crude enzyme (fraction I), stained with Coomassie Brilliant Blue; lane 2: approx. 100 ng of the enzyme after anion-exchange chromatography (fraction IV), silver-stain. Relative molecular masses of standard proteins are indicated.

polyglutamylase. While the bulk of the protein did not bind to the column, the enzyme stayed bound during the subsequent 0.25 M KCl wash. Glutamylase activity was eluted as a sharp peak by 20 mM MgATP and 0.5 M KCl. Affinity elution using free ligand alone (20 mM MgATP) led to a much broader elution profile of the enzyme and very low protein concentrations in the active fractions. Addition of 0.5 M KCl to the elution buffer helped to overcome this problem. The most active fractions from the affinity column displayed an approximately 500-fold increase in specific activity. The pooled fractions were approximately 200-fold enriched compared to the crude extract and the recovery of the enzyme was approximately 60%. Concentration of the sample by ultrafiltration prior to the following glycerol-gradient centrifugation resulted in significant losses (typically >50%) of total protein, even when the membrane devices were pretreated to improve recovery (see Materials and methods). Ion-exchange chromatography to concentrate the sample was not possible at this point because of the high concentration of KCl and ATP.

The use of glycerol-gradient centrifugation (Fig. 2B) as the next step had several advantages. First, glycerol stabilised the enzyme. The recovery after this step was typically greater than 70% while simply keeping fraction II at 4°C for 24 hours led to at least 50% loss in activity. Second, the gradient centrifugation provided another tenfold increase in specific activity as most of the protein is shifted towards higher S values. Third, KCl and ATP concentrations were reduced in preparation for the subsequent ion-exchange chromatography step (see below). Comparison with protein standards run in parallel tubes gave an S value of approx. 2.9 for the tubulin polyglutamylase. This corresponds to a molecular mass of about 38×10^3 for a globular protein. In good agreement with this value, analytical gel filtration of fraction II on Superdex 200 gave a native molecular mass of about 35×10^3 (R_s approx. 28Å; data not shown). However recovery of glutamylase activity in gel filtration was invariably low so that this step could not be used for preparative purification.

Fig. 2C shows the subsequent purification of fraction III (pooled fractions from one gradient tube, 2 ml volume) by anion-exchange chromatography on Mono Q. Most of the protein was eluted with 0.25 M NaCl and the activity was released as a sharp peak during the final wash with 0.9 M NaCl. Fraction III enzyme also bound to a cation-exchange column (Mono S) but was eluted as a broad peak with a bulk of contaminating proteins (not shown).

SDS-PAGE showed that the final preparation from the Mono Q column is dominated by a single polypeptide with an apparent molecular mass of about 40 kDa (Fig. 2D). Some minor contaminants are still present in this fraction, but their occurrence did not mirror the enzyme activity profile in the column fractions. Because of the presence of high salt the glutamylase activity is strongly inhibited in the final fractions and the salt cannot be efficiently removed by gel filtration or dialysis without nearly complete loss of the activity due to the very low protein concentration. An estimation from the protein profile shows that the Mono Q column leads to at least a fivefold enrichment of the enzyme, which indicates an overall purification factor of at least 10000. Because of the high salt concentration in fraction IV, most experiments to characterise

the enzyme were performed with the less pure fractions II or III.

General properties of tubulin polyglutamylase

ATP was required for the glutamylation reaction with an optimal concentration of 1-2 mM and could not be replaced by GTP or ATP- γ -S, a non-hydrolyzable ATP-analogue. Mg^{2+} was also absolutely required, with an optimal concentration of approx. 5 mM. At higher concentrations (>10 mM) both ATP and Mg^{2+} became inhibitory, leading to a 75% decrease of activity at 20 mM Mg^{2+} or to a 90% decrease at 10 mM ATP. The addition of 20% (v/v) glycerol to the reaction mixture led to a 10-15% increase in glutamylation activity, probably due to the stabilisation of the enzyme. Salts, such as NaCl or KCl, strongly inhibited the glutamylation reaction. For instance, 100 mM NaCl led to a >90% inhibition of polyglutamylase activity.

Incorporation rates for glutamic acid were consistently higher at pH 9.0 than at neutral pH. At the limiting glutamate concentration of 2.3 μ M, incorporation after 2 hours was approx. twofold higher at pH 9.0 than at pH 7.0. K_M values determined at pH 9.0 were approx. 0.3 μ M for tubulin and 0.8 mM for glutamic acid. Tubulin polyglutamylase showed a high specificity for L-glutamic acid. The presence of D-Glu, L-Tyr,

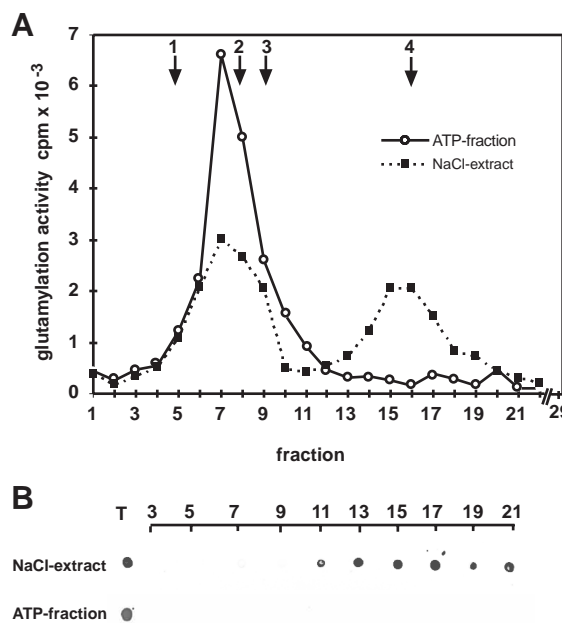


Fig. 3. Differential sedimentation behaviour of crude (NaCl extract) and partially purified tubulin polyglutamylase (ATP-fraction). (A) 300 μ l samples of crude (300 μ g of fraction I) and partially purified enzyme (50 μ g of fraction II) were loaded onto linear 10%-20% (v/v) glycerol gradients and centrifuged as described in Materials and methods. 10 μ l samples of each fraction were assayed for glutamylation activity. The positions of standard proteins are indicated at the top (see legend to Fig. 2B). (B) 1 μ l samples of the indicated fractions were spotted onto nitrocellulose and tubulin was detected with a monoclonal α -tubulin antibody. 1 μ g of phosphocellulose-purified pig brain tubulin served as control (T). Note the two peaks of activity (2.9 S and 7 S) in the crude enzyme and the single peak at 2.9 S in the enzyme from the ATP column. The larger peak in the crude enzyme contains tubulin, which is removed on the ATP-column.

L-Gly, L-Asp in 10- to 100-fold excess over L-Glu did not affect the glutamylation activity. Table 1 summarises the general properties of the enzyme.

Tubulin polyglutamylase forms a complex with $\alpha\beta$ tubulin

Fig. 3 illustrates the differential sedimentation behaviour of the crude enzyme after extraction from the microtubular cytoskeleton and of the partially purified enzyme after ATP-affinity chromatography. Glutamylation activity of the crude extract was resolved by glycerol-gradient centrifugation into two peaks. One corresponded to a *S* value of approx. 2.9 and the other to a species of approximately 7 *S* (corresponding to approx. 145 kDa for a globular protein). A dot assay (Fig. 3B) detected tubulin in the fractions of the second peak. After ATP-affinity chromatography the enzyme was free of tubulin (see Fig. 3B) and sedimented exclusively as the 2.9 *S* species. The value of 7 *S* suggests the existence of a 1:1 complex of the enzyme and the tubulin heterodimer (110 kDa). The complex is disrupted by the affinity chromatography (most of the tubulin is released during the 0.25 M KCl wash of the column). Only tubulin-free enzyme was observed in the subsequent density gradient centrifugation.

Glutamylation of brain tubulin by the *Crithidia* enzyme

The tubulin polyglutamylase recognised not only tubulin from trypanosomatids but also mammalian brain tubulin. Fig. 4 shows the glutamylation of microtubule protein from porcine brain by the partially purified *Crithidia* enzyme analysed by 2D-PAGE and ^3H -fluorography. The enzyme catalysed the incorporation of glutamic acid into both α - and β -tubulin. The label was preferentially found in the most acidic isoforms of both subunits. Longer exposure times also revealed labelling of the less acidic isoforms. While these results document the elongation of already existing side chains of brain α - and β -tubulins the resolution of the gels was not sufficient to identify unambiguously radioactively labelled monoglutamylated tubulin, i.e. the species expected for the first step in initiating a novel side chain on a previously non-glutamylated tubulin polypeptide. Identical results were obtained with the crude enzyme fraction from *Leishmania* and with MAP-free tubulin from porcine brain serving as substrate (not shown).

While mammalian brain tubulin was effectively polyglutamylated by the *Crithidia* enzyme, tubulin from HeLa cells was a poorer substrate. After a 2 hour incubation, glutamylation of HeLa tubulin reached only 15-20% of the

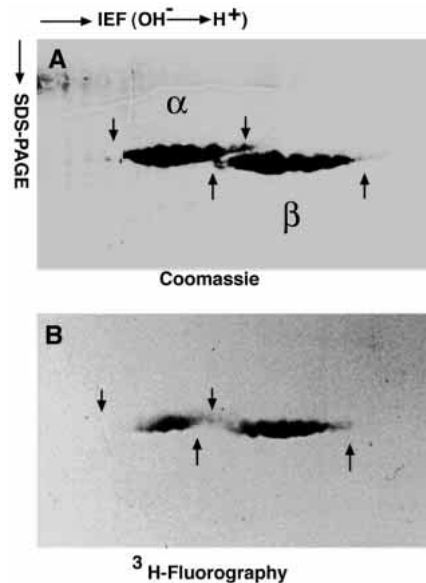


Fig. 4. Two-dimensional gel analysis of pig brain tubulin treated with *Crithidia* polyglutamylase. 50 μg brain microtubule protein was incubated with partially purified enzyme (5 $\mu\text{g}/\text{ml}$ fraction II) at a limiting glutamate concentration (2.3 μM [^3H]glutamic acid) in the standard assay. The stained gel (A) was exposed to X-ray film for 48 hours (B). Only the tubulin-containing region of the gel is shown. The arrows allow an easier orientation between A and B. Note that the label is preferentially found towards the acidic side of both α - and β -tubulin variants.

value obtained for brain tubulin. HeLa cell tubulin is mostly unglutamylated but contains low levels of monoglutamylated α - and β -tubulin (Bobinac et al., 1998; Regnard et al., 1998).

Elongation of oligoglutamyl side chains of carboxy-terminal peptides

To further investigate the substrate specificity of the tubulin polyglutamylase we tested the synthetic peptides corresponding to the carboxy-terminal 15 and 19 residues of tyrosinated $\alpha 1/\alpha 2$ and $\beta 2$ (class II) mammalian brain tubulins. The peptides were used in normal form and as derivatives carrying an oligoglutamyl side chain of four residues on previously established positions, i.e. Glu-445 for α and Glu 435 for β (Eddé et al., 1990; Rüdiger et al., 1992). The last three glutamic acid residues of the side chain were connected via normal α peptide bonds.

Fig. 5 shows that the branched α peptide with a four-residue glutamyl side chain showed a significantly greater ability to reduce tubulin glutamylation than the unmodified α peptide. The branched peptide gave a 50% reduction of the activity at a concentration of approx. 50 μM , which is approx. 25-fold molar excess over tubulin in this reaction. Because of this effect we tested the α peptides as potential substrates and characterised the products by HPLC with a mass spectrometric analysis of the resulting fractions. While the unbranched peptide did not incorporate glutamic acid (data not shown) the branched peptide was recognised as substrate. The polyglutamylase incorporated up to three additional glutamyl residues (Fig. 6B). Automated Edman degradation showed that these residues were added to the already existing side chain,

Table 1. Properties of *Crithidia* tubulin polyglutamylase

Apparent molecular mass	~ 40 kDa (SDS-PAGE)
<i>S</i> -value	2.9 <i>S</i> (free enzyme) 7 <i>S</i> (complex with tubulin)
Nucleotide dependence	ATP (GTP excluded)
Stereospecificity	L-glutamic acid (D-glutamic acid excluded)
pH optimum	approx. pH 9.0
K_M	approx. 0.3 μM (tubulin) approx. 0.8 mM (glutamic acid)
Inhibition	by salt (NaCl, KCl) by higher concentration of Mg-ATP

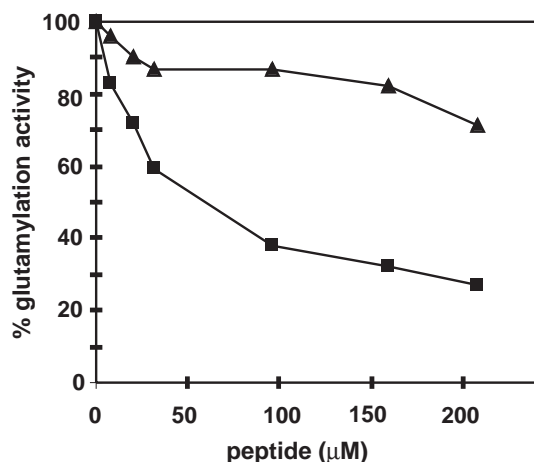


Fig. 5. Inhibition of tubulin polyglutamylation by synthetic α peptides. Glutamylation of brain tubulin with crude enzyme (10 μ g of fraction I) was competed with a synthetic peptide representing the last 15 residues of tyrosinated brain α tubulin (filled triangles), and the corresponding branched peptide (filled squares) carrying a side chain of four glutamyl residues on the glutamic acid in position 9. Standard assays were performed at the indicated peptide concentrations and TCA-precipitable radioactivity was measured as described in Materials and methods. Note the much stronger inhibition by the branched peptide.

since no additional novel side chain could be detected. In agreement, treatment of the enzymatically elongated peptide with carboxypeptidase A removed the full complement of the terminal tyrosine. Similarly the branched β peptide increased by up to five glutamyl residues along the side chain (Fig. 6D) while the normal β peptide was not used as substrate (data not shown).

In these experiments we noted that the α peptides also gave rise to components with a mass increment of 79. Partial acid hydrolysis identified Ser-PO₄. Thus after the ATP-affinity chromatography the enzyme preparation contains a protein kinase that phosphorylates the single serine of the peptides, which corresponds to Ser-439.

DISCUSSION

We have established a method that results in a nearly homogenous preparation of tubulin polyglutamylase. Although isolated from *Crithidia*, a member of the trypanosomatids, the protozoan enzyme accepts mammalian brain α - and β -tubulins as substrates. The enzyme seems exclusively bound to the stable microtubular cytoskeleton, which is isolated by Triton extraction, in agreement with the notion that microtubules are the preferred substrate of the polyglutamylase (Audebert et al., 1993; Regnard et al., 1998). This observation greatly facilitated the purification since the cytosol, which shows no activity, is readily removed. A further advantage of the use of trypanosomatids is the finding that the enzyme is efficiently removed from the microtubules by a 0.25 M salt wash. Glycerol-gradient centrifugation of the resulting soluble enzyme showed that some of the polyglutamylase sediments as free monomer at 2.9 S, while the remainder formed a complex at 7 S with $\alpha\beta$ tubulin (Fig. 3). This complex is

dissociated upon ATP-affinity chromatography, which provides a highly enriched preparation in which all of the polyglutamylase sediments at 2.9 S in the subsequent step. A further purification can be reached by Mono Q chromatography. Although the purified enzyme loses activity at 4°C and at room temperature, especially when present at high dilution, conditions have been found to stabilise the enzyme and to store it at -80°C.

The *Crithidia* tubulin polyglutamylase has a molecular mass of 38×10^3 in glycerol-gradient centrifugation and SDS-PAGE shows that it consists of a single polypeptide chain (Fig. 2). Interestingly tubulin tyrosine ligase, which also catalyses the ATP-dependent synthesis of a peptide bond, is a monomer with a molecular mass of 43×10^3 (Schröder et al., 1985; Ersfeld et al., 1993). Thus one wonders whether the enzymes responsible for all three tubulin modifications, which involve the ATP-dependent formation of peptide bonds (tyrosination, glutamylation and glycylation), belong to an enzyme family. Interestingly the sequence of the ligase (Ersfeld et al., 1993) shares a fold with the glutathione synthetase ADP-forming family of enzymes (Dideberg and Bertrand, 1998). Future sequence data will decide whether this also holds for the tubulin polyglutamylase. We note, however, already that ATP-affinity chromatography performs very well in both the case of tubulin tyrosine ligase (Flavin and Murofushi, 1984; Schroeder et al., 1985) and tubulin polyglutamylase (see Results) when the ATP is coupled to Sepharose via a spacer of sebacic acid dihydrazide, while other affinity resins show poor performance (see Results).

Interestingly the *Crithidia* enzyme acts not only on brain tubulin (Fig. 4) but also recognises, although at low efficiency, the branched carboxy-terminal peptides of the major brain α - and β -tubulins as substrates. The polyglutamylase elongates the oligoglutamyl side chain by up to 3 (α) and 5 (β) additional glutamyl residues (Fig. 6). In contrast the corresponding peptides without the oligoglutamyl side chain are not accepted as substrates in the standard assay. Thus the synthesis of the isopeptide bond resulting in the monoglutamylated derivative requires either the tubulin molecule itself and/or a second enzymatic activity. Future experiments have to distinguish between these possibilities (see below).

Regnard et al. (1998) recently described a polyglutamylase activity enriched from brains of 3-day-old mice. The enzyme sediments at 10 S (apparent molecular mass 360 kDa) and comprises in SDS-PAGE numerous polypeptides among which that (or those) corresponding to tubulin polyglutamylase cannot be identified. The major polypeptides at 110 and 130 kDa were ruled out as glutamylase subunits because they do not bind to microtubules. A detailed protein chemical analysis of the glutamylated brain tubulin peptides showed that the murine brain glutamylase not only extends already existing glutamyl side chains but can also catalyse the synthesis of the isopeptide bond on a nonglutamylated tubulin molecule. The enzyme preparations from mouse brain and *Crithidia* share a number of typical properties but differ in other aspects. Both enzymes show similar K_M values for tubulin and glutamic acid, are progressively inhibited by increased concentrations of monovalent cations and Mg-ATP and have higher activity at pH 9.0 than at pH 7.0. A possible reason for the high pH optimum could be that the free amine of glutamate (pK 9.7) is the substrate in the reaction. The

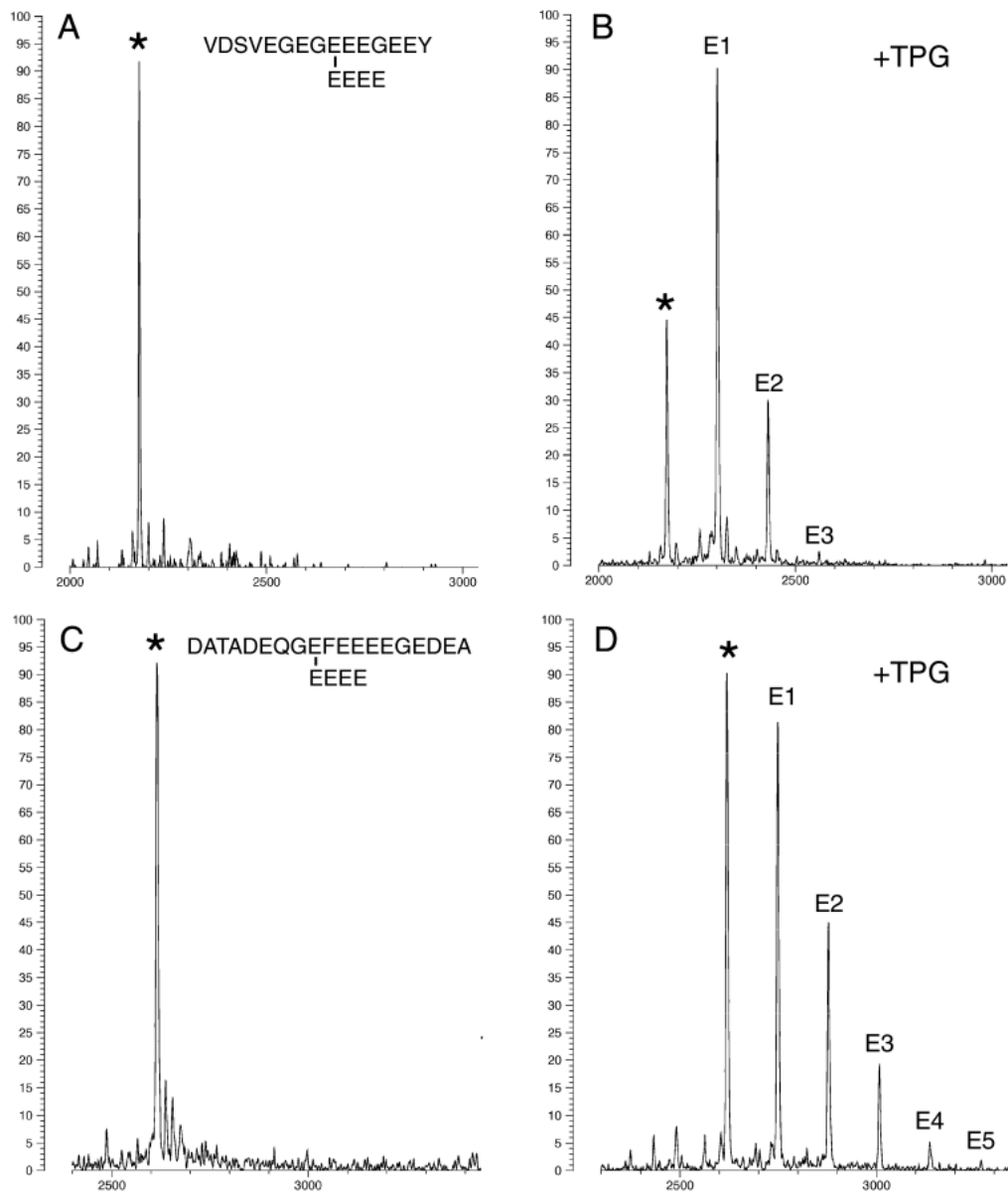


Fig. 6. Branched synthetic peptides covering the carboxy-terminal end of brain α - and β -tubulins are substrates of *Crithidia* tubulin polyglutamylase (TPG). Mass spectra of the α peptide (carboxy-terminal 15 residues with an oligoglutamyl side chain of four residues on position 9) and the β peptide (carboxy-terminal 19 residues with a side chain of four residues on position 9) are given in A and C. Mass spectra of samples of the products (+TPG) are shown in B (α) and D (β), respectively. The original peptides are marked by an asterisk. The number of additional glutamyl residues incorporated into the side chain is indicated as E1 to E5. Assays were performed with 0.1 μ g of fraction II at pH 9.0 for 16 hours to enhance polyglutamylation.

much higher molecular mass of the brain enzyme compared to our preparation could be due to a complex combining several activities. The presence of a complex may also explain why the brain enzyme could not be purified by ATP-affinity chromatography and ion-exchange chromatography, which perform very well with polyglutamylase from *Crithidia*. Because of a different stereochemistry synthesis of a monoglutamylated side chain, which involves an isopeptide bond, an elongation based on α peptide bonds could require distinct enzymes. Although a preference of α peptide bonds along the side chains is indicated in several studies (Redeker et al., 1991, 1996; Rüdiger et al., 1999) the occasional isopeptide bond requiring an additional enzyme cannot be dismissed (Wolff et al., 1994). In addition there could be different enzymes for α - and β -tubulins and even for different tubulin isotypes (Regnard et al., 1998). Finally it is unclear whether polyglutamylation at more than one site of the same

tubulin molecule requires different enzyme forms. Multiple glutamylation sites have been documented in both α - and β -tubulin from *Trichomonas* (Schneider et al., 1998) and we have recently found that $\alpha 4$ tubulin from pig brain carries side chains at Glu 443 as well as at Glu 445 (our unpublished results).

Currently we do not know whether our preparation of tubulin polyglutamylase from *Crithidia*, which effectively glutamylates α - and β -brain tubulin, is composed of several distinct isozymes. Rather than trying to separate such forms we aim at cDNA cloning, based either on peptide sequence information or on antibodies which bind to the enzyme. Expression of complete cDNA clones in *E. coli* or in insect cells, as well as a detailed analysis of the purified recombinant protein, should show which glutamylation activities can be assigned to the single polypeptide chain. This approach can be extended to isolate corresponding cDNA clones from

Leishmania or *Trypanosoma*, where gene replacement is possible (ten Asbroek et al., 1990; Cruz et al., 1991) to analyse the function of tubulin polyglutamylated. In this respect trypanosomatids are particularly attractive, since their microtubules are extensively polyglutamylated (Schneider et al., 1997).

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Note added in proof

We recently found that the enzyme adds up to 6 additional glutamate residues to the branched α -peptide.