FEBS 17455 FEBS Letters 393 (1996) 27–30

Polyglycylation of tubulin in the diplomonad *Giardia lamblia*, one of the oldest eukaryotes

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Received 16 July 1996

Abstract We have searched for post-translational modifications in tubulin of the diplomonad *Giardia lamblia*, which is a representative of the earliest branches in eukaryotic evolution. The carboxyterminal peptide of α-tubulin was isolated and characterized by automated sequencing and mass spectrometry. Some 60% of the peptide is unmodified, while the remainder shows various degrees of polyglycylation. The number of glycyl residues in the lateral side chain ranges from 2 to 23. All peptide species encountered end with alanine–tyrosine, indicating the absence of a detyrosination/tyrosination cycle. We conclude that tubulin-specific polyglycylation could be as old as tubulin and axonemal structures.

Key words: Flagella; Polyglutamylation; Polyglycylation; Post-translational modification; Tubulin; Tyrosination

1. Introduction

The αβ-tubulin heterodimer, the structural unit of microtubules, is the target of various post-translational modifications. Some of these, like the acetylation of lysine-40 in certain α-tubulins [1,2] and some phosphorylation sites in the carboxyterminal region [3,4] are more general post-translational modifications, while others seem unique to tubulins. Thus the carboxyterminal tyrosine of certain α-tubulins is subject to a detyrosination/tyrosination cycle based on a carboxypeptidase and the well-characterized tubulin-tyrosine ligase [5,6]. After loss of the tyrosine and penultimate glutamic acid residue the resulting tubulin is no longer a substrate for tubulin-tyrosine ligase [7–9]. Two other unique post-translational modifications involve the presence of a polyglutamyl- or polyglycyl side chain attached via an isopeptide bond to a particular glutamic acid residue in the carboxyterminal region of α- and β-tubulin. The exact position of this modified residue has been identified in all brain tubulins [3,8,10–13]. The more recently discovered polyglycylation has been documented for α- and β-tubulin from the ciliary axonemal microtubules of the protist *Paramecium* [14] and from the sperm axonemal microtubules of mammals [15] (Plessmann, U. and Weber, K. unpublished results) and sea urchins [16]. Thus all post-translational modifications unique to tubulin involve the negatively charged carboxyterminal 10 to 15 residues [12,15]. Since the molecular functions of these post-translational modifications are still a matter of debate it is interesting to search for their evolutionary origin because they are unique to tubulin, a typical eukaryotic protein.

Based on ultrastructural characteristics and several molecular phylogenies there is general agreement that the diplomonads were among the first branches which emerged from the eukaryotic tree. Diplomonads, like other Archezoa, are thought to have arisen before the acquisition of mitochondria and to have retained many primitive features of the first nucleated cells [17–21]. *Giardia lamblia* is a particularly well-characterized diplomonad. Its cytoskeleton is dominated by microtubules. *Giardia* has eight flagellar axonemes and a large disc cytoskeleton which consists of microtubules and the microribbons, which arise from β-giardin [22,23]. Here we report an analysis of the post-translational modifications of α-tubulin from *Giardia* based on the characterization of its carboxyterminal peptide.

2. Materials and methods

2.1. Cells, cytoskeletal preparations and tubulin isolation

The origin of the *Giardia lamblia* isolate WB has been described [24]. Trophozoites were cultivated in TY-S-33 medium with antibiotics [25]. Several cultures of *Giardia l.* were harvested, washed in phosphate-buffered saline and frozen as pellets in liquid nitrogen. Frozen pellets (total 1.5×10^9 cells) were suspended in 50 ml of Tris-HCl, pH 8.3, 2 mM EDTA, 2 mM DTT, 0.5 mM ATP, 2 mM $MgSO_4$, 150 mM KCl containing 0.5% Triton X-100. Detergent extraction of cytoskeletons was as described [26]. Cytoskeletons were resuspended in SDS sample buffer, boiled and subjected to SDS-PAGE using a preparative 10% gel. After staining with Coomassie brilliant blue the major band corresponding to α- and β-tubulin was excised, washed with water and frozen at −70°C until use.

2.2. Isolation and characterization of the carboxyterminal peptides of α-tubulin

Tubulin present in dye-stained gel pieces was electrophoretically concentrated into a small band by the agarose gel concentration system [27]. Treatment with endoproteinase LysC (Boehringer, Mannheim, Germany) was at 3 μg/ml in 0.1 M Tris-HCl, pH 8.5, 5% in acetonitrile, for 16 h at 37°C. The digest was recovered in buffer A (20 mM sodium phosphate, pH 7) by the use of a SMART (Pharmacia, Uppsala, Sweden) fast desalting column and separated on a Mono Q column (1.6×50 mm) equilibrated with buffer A with a 2.4 ml salt gradient (0–0.5 M NaCl in buffer A) followed by a 0.9 M salt wash. The flow rate was 100 μl/min. The elution profile was monitored by absorption at 214 nm and conductance measurement. Fractions of 100 μl were collected. Appropriate peak fractions were processed by reverse-phase HPLC on a Vydac 218 TP51 column. Peptides were eluted with a 2.4 ml linear gradient from 10 to 80% solvent B at a flow rate of 40 μl/min. Solvent A was 0.1% TFA. Solvent B was 70% acetonitrile in 0.08% TFA. Elution profiles were monitored by absorption at 214 nm. Peak fractions were collected manually and characterized by mass spectra recorded with a KRATOS MALDI 3 time of flight mass spectrometer (Shimadzu, Duisburg, Germany). The matrix was α-cyano-4-hydroxy-cinnamic acid in 30% acetonitrile, 0.06% TFA. Spectra of negative ions were recorded in the linear mode. Calibration was with bovine insulin, α-melanocyte stimulating hor-

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Abbreviations: HPLC, high-performance liquid chromatography; MALDI, matrix-assisted laser desorption ionization; TFA, trifluoroacetic acid

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PII S0014-5793(96)00848-4

Fig. 1. SDS-PAGE analysis of *Giardia* cytoskeletal preparation. *Giardia* cells were extracted with 0.5% Triton X-100 and the insoluble residue was subjected to SDS-PAGE using a 10% gel. Note the strong tubulin band marked by a star and the lower molecular weight giardins.

mone and synthetic peptides. Automated Edman degradation used instruments with online phenylthiohydantoin amino acid analysis.

3. Results

Giardia cells were extracted with a buffer containing Triton X-100 (see Section 2). SDS-PAGE showed that the resulting cytoskeletal preparation is dominated by tubulin and the giardins (Fig. 1; [26]). Preparative SDS-PAGE was used to purify *Giardia* tubulin. The Coomassie brilliant blue-stained band was excised and an aliquot of the gel fragments was processed for digestion with endoproteinase LysC. The digest was chromatographed on a small Mono Q column and all later emerging peaks were subjected to reverse-phase HPLC. All peptide containing fractions from the HPLC columns were characterized by mass spectrometry. Major fractions were also analyzed by automated Edman degradation.

The carboxyterminal peptide of *Giardia* α-tubulin eluted at several positions from the Mono Q column. The major amount, eluting around fraction 26, provided several peaks in subsequent HPLC. Some 50% of the peptide, which eluted later from the HPLC column showed a mass of 2652 (Fig. 2A). This value is in excellent agreement with the sequence DYEEIGAETLGDGEGEDME*E*DDAY established by automated sequencing (calculated value 2652). Around 40% of the peptide eluting in two additional fractions showed polyglycylation. In one fraction the number of extra glycyl residues ranged from 0 to 6. Components with 0, 2 and 3 extra glycyl residues were the major constituents in this fraction (Fig. 2B).

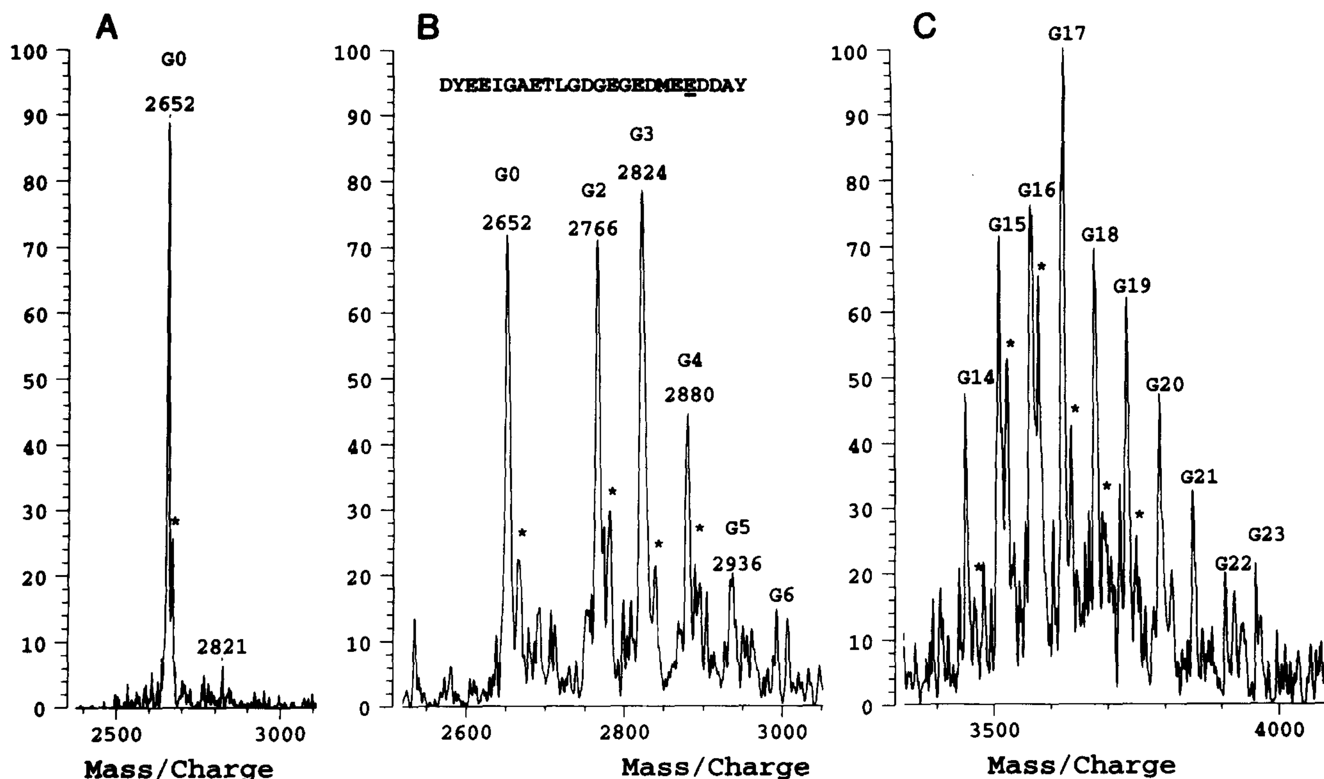

Fig. 2. Mass spectra of the carboxyterminal peptides of *Giardia* α-tubulin generated by endoproteinase LysC. (A) The MALDI mass spectrum of the unmodified peptide (marked G0). Its mass/charge of 2652 fits exactly the amino acid sequence established by automated Edman degradation (top line in B). The minor peak marked by a star has a mass increment of 16 and corresponds to a fraction of the peptide in which the methionine was oxidized to the methionine sulfoxide. (B) Fraction of the peptide containing the unmodified species and several glycylated components which are marked as G2–G6 according to the number of additional glycyl residues. Stars are used as in (A). The sequence of this preparation (top line) was the same as established before, except for a lower yield at the position of the second glutamic acid (underlined) following the methionine (see Section 3). (C) The MALDI spectrum of a highly glycylated peptide, which was recovered in low yield. Note that the number of additional glycine residues ranges from 14 to 23 (peaks G14 to G23). Stars are used as above.

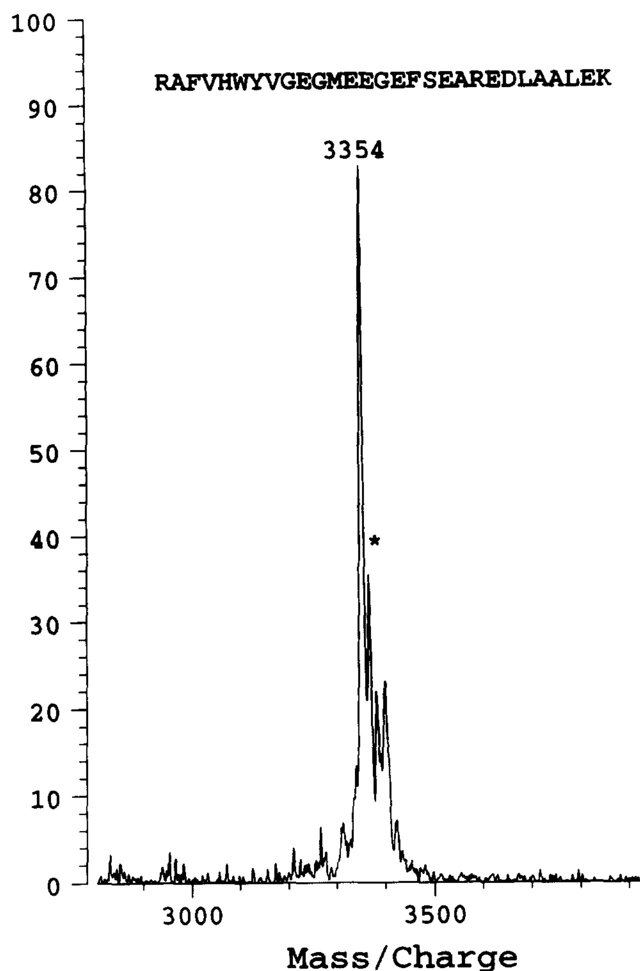

Fig. 3. Mass spectrum of the penultimate carboxyterminal peptide of *Giardia* α-tubulin generated by endoproteinase LysC. Its mass/charge of 3354 fits exactly the amino acid sequence established by automated Edman degradation (top line). The star indicates a fraction of the peptide in which the methionine was oxidized to methionine sulfoxide.

In the earlier eluting fraction the number of glycyl increments ranged from 6 to 16. Sequencing of the former fraction, which also contained some unmodified peptide, yielded the same sequence as above, but the second glutamic acid residue (underlined in the sequence) following the unique methionine showed a reduced yield compared to the preceeding and following residue. Thus the oligoglycyl chains may be connected to this particular glutamic acid residue via the isopeptide bond.

A more heavily glycylated derivative of the carboxyterminal α-tubulin peptide eluting around fraction 19 from the Mono Q column was a minor component. Here the number of extra glycyl residues ranged from 14 to 23 and the main component showed 17 glycines for the side chain (Fig. 2C). All derivatives of the carboxyterminal peptide end with a tyrosine preceeded by an alanine (see Section 4). The cDNA sequence of *Giardia* β-tubulin (accession number P05304; [28]) predicts that LysC digestion should yield a 53 residue carboxyterminal peptide. In agreement with earlier work on bull sperm tubulin [15] we assume that this peptide is not recovered by our procedures.

A small amount of peptide material eluting very late from the Mono Q column (around fraction 31) gave rise to several peaks upon HPLC analysis. Two of these showed in mass spectrometry components related by one and two glutamic acid residues. Since their mass values seem unrelated to those of the α-tubulin peptides we do not know whether they reflect a glutamylated tubulin. Such a possibility can only be explored once sufficient material for sequence analysis is available.

Since for diplomonads only a partial α-tubulin sequence (*Hexamita inflata*) is known [21] we completed the carboxyterminal region by the characterization of the penultimate peptide of *Giardia* which shows no modifications (Fig. 3). Its sequence starts at the last lysine in the partial *Hexamita* sequence (accession number U37080) and covers 29 residues. These are identical with the corresponding sequence (residues 402 to 430) of α-tubulin from *Trypanosoma brucei* ([29]; accession number P04106). The following carboxyterminal peptide of *Giardia* (see above) is by three residues longer than the *Trypanosoma* sequence.

4. Discussion

Using sequence analysis and mass spectrometry we have shown that a sizeable fraction of the carboxyterminal peptide of *Giardia* α-tubulin is polyglycylated. The number of glycyl residues in the lateral side chain ranges from 2 to 23 with the species containing 2 to 6 glycyl residues accounting for the majority of the peptide species. Although the *Giardia* β-tubulin was not analyzed, previous experience with axonemal microtubules from the ciliate *Paramecium* and the sperm flagella of sea urchin show that both α- and β-tubulin are polyglycylated [14,16]. An earlier report for bull sperm [15] documented only β-tubulin glycylation but subsequent work showed that the glutamylated α-tubulin can carry up to 23 glycyl residues (Plessmann, U. and Weber, K. unpublished results). Given the fact that tubulin polyglycylation has so far emerged primarily in axonemal structures (above references and [30]) we assume that this will also be the case in *Giardia* which has eight flagellar axonemes. Whether the large disk cytoskeleton consisting of microtubules and the giardin microribbons [26] also contains glycylated tubulin is currently not known. An answer may be expected from the use of recently described antibodies specific for highly glycylated tubulins [30].

In spite of the considerable heterogeneity of the carboxyterminal peptide of α-tubulin due to oligoglycyl chains of various length all derivatives end with the sequence D-A-Y, which after detyrosination would not be accepted as substrate by mammalian tubulin-tyrosine ligase [9]. This result and the lack of any detyrosinated α peptide argue that *Giardia* tubulin is not subject to a detyrosination/tyrosination cycle. Here we note that α-tubulin of *Trypanosoma brucei*, a member of the Kinetoplastida, is, in line with its sequence, subject to such a cycle [31,32] while α-tubulins from some ciliates show sequences not compatible with the normal cycle (see for instance [14]). The reason for this interesting variability is not known. Although a polyglutamylated α-tubulin peptide of *Giardia* was not encountered we cannot exclude the possibility that this post-translational modification exists at low abundancy and possibly involves another tubulin isotype (see Section 3). Since cytoskeletal preparations were performed on previously frozen cells, normal cytoplasmic microtubules may not have been recovered in the insoluble residue used for tubulin purification. In addition some modified tubulins can

show abnormal mobility and poor staining in SDS-PAGE [30]. Thus future experiments on *Giardia* should make use of antibodies specific for some polyglutamylated tubulins [33].

The combined results show that at least one post-translational modification unique to tubulin is already present in the diplomonad *Giardia*, which belongs to the earliest branches in eukaryotic evolution. Polyglycylation seems connected to axonemal organization (see above) and *Giardia* has already typical axonemes. Polyglycylation is thought to occur after axonemes are formed and may be involved in membrane connections [34] and a structural distinction between A and B tubules of the outer doublet microtubules [16].

Acknowledgements: This work was supported by Grants 30-37607-93 (to N.M.), 31-40701-94 (to A.S.) of the Swiss National Foundation, by a fellowship of the 'Prof. Dr. Max Cloëtta' Foundation (to A.S.) and funds of the Max Planck Society (to K.W.)

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