

Posttranslational modifications of α - and β -tubulin in *Giardia lamblia*, an ancient eukaryote

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Abstract Tubulin of *Giardia lamblia*, a representative of the oldest eukaryotes, was screened for posttranslational modifications. Mass spectrometry of the carboxy-terminal peptides documents a large number of variants. Both α - and β -tubulin show polyglycylation with up to 20 and 15 extra glycylation residues respectively. Minor variants show a low level of glutamylation without or with glycylation. The glutamylation-specific antibody GT335 detects α - and β -tubulin in immunoblots. The terminal tyrosine is fully retained in α -tubulin, which is completely acetylated at Lys-40. Thus except for the detyrosination/tyrosination cycle all posttranslational modifications known for higher eukaryotes are already present in *Giardia*.

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Key words: Polyglutamylation; Polyglycylation; Posttranslational modification; Tubulin; Tyrosination; *Giardia lamblia*

1. Introduction

The structural unit of microtubules is the $\alpha\beta$ tubulin heterodimer which is usually encoded by small multigene families for both α - and β -tubulin. Tubulins are subject to a number of posttranslational modifications. Some α -tubulins are acetylated at Lys-40 [1] and certain β -tubulin isotypes show phosphorylation of a serine in the carboxy-terminal region [2–4]. In addition there is a set of posttranslational modifications which seems restricted to tubulins. Certain α -tubulins show a cycle for the carboxy-terminal tyrosine based on a carboxypeptidase activity and a re-addition due to tubulin-tyrosine ligase [5]. The latter enzyme has been purified and cloned from mammalian brain [6]. Further excision involving the penultimate glutamyl residue leads to a tubulin which is no longer a substrate for the ligase [7,8]. Two modifications are common to α - and β -tubulin. Polyglutamylation [9] and polyglycylation [10] involve the addition of several residues to the γ -carboxyl group of a glutamyl residue located within the carboxy-terminal 12 residues. Thus all tubulin-specific modifications involve the carboxy-terminal region which is usually rather acidic in nature. Polyglycylation has so far been documented only for axonemal microtubules of cilia and flagella. Although found in protists such as *Paramecium* [10] and *Giardia* [11], in sperm of sea urchins [12–14] and bull [15,16] it is conspicuously absent from *Trypanosoma* [17].

Molecular phylogenies covering ribosomal RNA [18], actin [19], α -tubulin [20] as well as the elongation factors 1 α and 2 [21] all show that diplomonads like *Giardia lamblia* probably reflect the oldest branch of the eukaryotic tree. *Giardia* has

eight flagellar axonemes and a large disc consisting of microtubules and microribbons [22,23]. Thus cytoskeletons of *Giardia* can be used to decide which posttranslational modifications of tubulin are already present in an ancient eukaryote. Here we show that *Giardia lamblia* has almost the entire complement of tubulin-specific modifications. We discuss that polyglutamylation is a widespread tubulin modification while polyglycylation is restricted to some but not all axonemal microtubules.

2. Materials and methods

2.1. Cells, cytoskeletal preparations and tubulin isolation

Giardia culture, detergent extraction with 0.5% Triton X-100 and SDS-PAGE of the resulting cytoskeletal preparation were as described [11]. After staining with Coomassie brilliant blue the dominant tubulin band corresponding to α - and β -tubulins was excised, briefly washed with water and frozen at -70°C until use.

2.2. Isolation and characterization of the carboxy-terminal peptides

The carboxy-terminal peptides of β -tubulin were isolated from a CNBr digest. Stained gel fragments containing tubulin were processed for in situ CNBr treatment [15]. After lyophilization the digest was recovered in buffer A (20 mM Tris-HCl, pH 8) by a SMART fast desalting column and separated on a TSK gel DEAE-NPR column (Tosoh, Stuttgart, Germany) as described [10]. The column dimensions were 4.6×35 mm. The flow rate was 500 $\mu\text{l}/\text{min}$ and fractions of 200 μl were collected. The elution profile was monitored by absorption at 214 nm. Appropriate fractions of the acidic peptides were processed by reverse-phase HPLC on a Vydac 218 TP 51 column as described [11]. The carboxy-terminal peptides of α -tubulin were isolated from a digest with endoproteinase LysC and processed through Mono Q and HPLC [11].

All peak fractions from the HPLC runs were characterized by mass spectra recorded with a Kratos MALDI 3 or 4 time of flight mass spectrometer (Shimadzu, Duisburg, Germany). The matrix was α -cyano-4-hydroxycinnamic acid in 30% acetonitrile, 0.06% TFA. Spectra were recorded in the linear negative mode. Calibration was with bovine insulin, α -melanocyte stimulating hormone and synthetic peptides. Major peptide peaks were analyzed by automated Edman degradation using instruments with on-line phenylthiohydantoin amino acid analysis.

The carboxy-terminal sequence of the unmodified β -tubulin peptide was obtained by carboxypeptide digestion monitored by mass spectrometry using conditions similar to those given by Bonetto et al. [24]. Digestion was in 50 mM sodium citrate buffer, 10% acetonitrile, pH 5.5 with 0.4 μg carboxypeptidase Y (Boehringer, Mannheim, Germany) in 7 μl at room temperature. Aliquots removed after 30, 60, 130 and 230 min were subjected to mass spectrometry.

2.3. Acetylation of Lys-40 in α -tubulin

An aliquot of a cytoskeletal preparation was subjected to SDS-PAGE using 6 M urea in the solution for the running gel. This procedure separates α - and β -tubulin [17]. The α -tubulin containing gel fragments (upper band) were subjected to in situ fragmentation by CNBr, separated by SDS-PAGE and electrophoretically blotted onto a poly(vinylidenedifluoride) membrane. A CNBr fragment with

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an apparent M_r of 14000 was subjected to automated sequencing, which resolves the phenylthiohydantoin derivatives of lysine and *N*-ε-acetyl-lysine [1].

2.4. Immunoblotting

SDS-PAGE of the cytoskeletal residue was performed in the presence of 6 M urea in the separation gel to separate α- and β-tubulin. Corresponding blots were analyzed with the murine monoclonal antibody DM1A (Sigma, Deisenhofen, Germany), a general antibody for α-tubulins, and the murine monoclonal antibody GT 335 [25], kindly provided by Dr. B. Eddé. The second antibody was peroxidase-labelled rabbit anti-mouse antibody (Dako, Hamburg, Germany).

3. Results

SDS-PAGE of *Giardia* cells extracted by Triton X-100 showed that the cytoskeletal preparation is dominated by tubulin and the lower molecular weight giardins [11,22,23,26]. Preparative SDS-PAGE was used to isolate the *Giardia* tubulin. The Coomassie brilliant blue-stained tubulin band was excised and aliquots of the gel fragments were processed to isolate the carboxy-terminal peptides.

3.1. Glycylated and glutamylated β-tubulin variants

After in situ treatment with CNBr the mixture of tubulin fragments was chromatographed on a small DEAE column. The various acidic peptides eluting late from the column were subjected to HPLC as described in Section 2. All peaks from the HPLC experiments were monitored by mass spectrometry and the major peaks were also subjected to automated Edman degradation. Fig. 1 summarizes the large number of variants of the carboxy-terminal β-tubulin peptide. Its sequence of 31 residues was established on a fraction containing essentially unmodified peptide and is given at the top of the figure. Ex-

cept for the last two residues this sequence fits the carboxy-terminal sequence following methionine-415 predicted by cDNA cloning. The terminal sequence has been given as FGDE [27] and FGDEQ (accession number P05304) while we find FGDEYA by direct automated sequencing. Our sequence is independently supported by carboxypeptidase Y digestion monitored by mass spectrometry. A time course experiment shows that the terminal alanine (A) is preceded by tyrosine (Y). Lack of further digestion is in line with the preceding acidic residues.

The major fractions of the β-peptide are the unmodified form and the series of glycylated derivatives ranging from 1 to 15 extra glycyl residues (Fig. 1). In this series of polyglycylated variants the main components have 8–12 extra glycyl residues. Less abundant variants show either 1 or 2 additional glutamyl residues with or without 1–3 extra glycyl residues (Fig. 1C). These more acidic species eluted as the last peak from the DEAE column. We also observed a series of variants containing 6–12 extra glycyl residues in addition to a single extra glutamyl residue (Fig. 1D). A rough estimate of the relative abundance based on the peak heights of the various HPLC chromatograms and the distribution observed by mass spectrometry indicates that the unmodified and the glycylated series of peptides are the major components. The glutamylated peptides and the peptides containing both modifications reflect minor species.

3.2. Glycylated and glutamylated α-tubulin variants

The carboxy-terminal α-tubulin variants were obtained from a digest of the tubulin band with the endoproteinase LysC separated on a Mono Q column. The various acidic peptide peaks eluting late from the anion exchange column

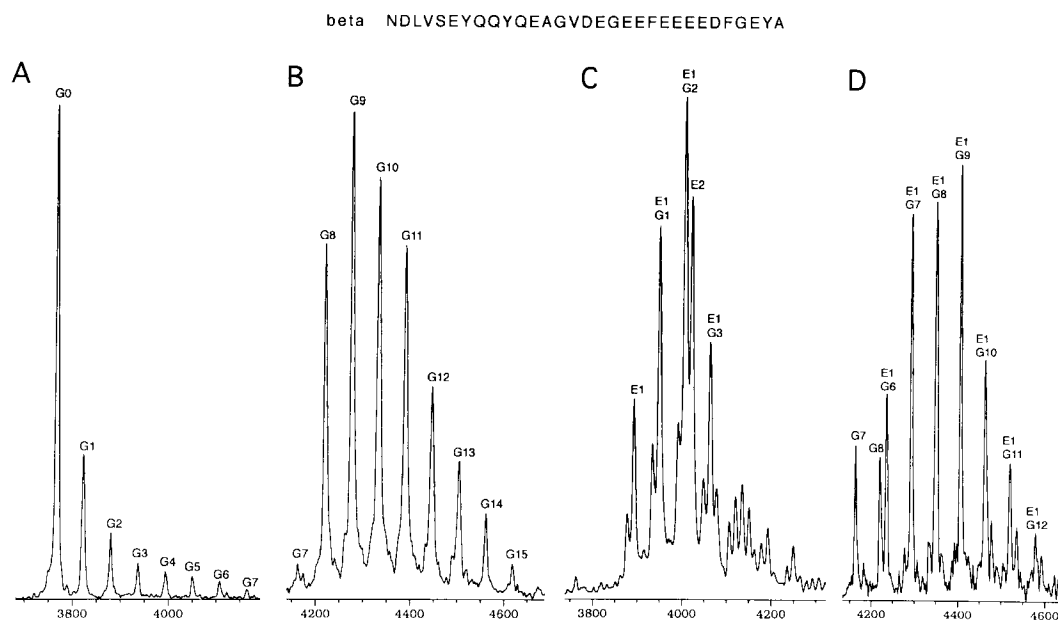


Fig. 1. Mass spectra of the carboxy-terminal peptides of *Giardia* β-tubulin generated by CNBr cleavage. A: Mass spectrum of a fraction containing the unmodified species (G0) and several glycylated components which are marked G1–G7 according to the number of additional glycyl residues. The amino acid sequence obtained by Edman degradation is given at the top. B: Fraction of the peptide showing higher levels of glycylated. The number of extra glycyl residues ranges from seven (G7) to 15 (G15). C: Mass spectrum of a fraction of the peptide in glutamylated and glycylated form. The number of glutamyl residues is one or two (E1, E2). Some species carry in addition 1–3 extra glycyl residues marked G1–G3. D: Peptide variants with one additional glutamyl residue (E1) and 6–12 extra glycyl residues (G6–G12). The left part of the spectrum shows two variants with seven and eight glycyl residues only.

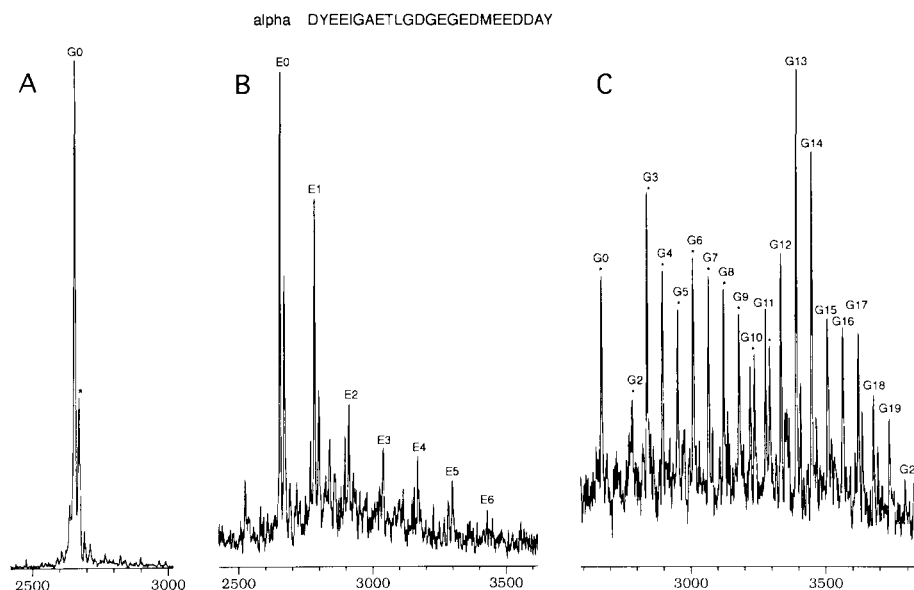


Fig. 2. Mass spectra of the carboxy-terminal peptides of *Giardia* α -tubulin generated by endoproteinase LysC. A: Mass spectrum of the unmodified peptide (marked G0). The sequence obtained by Edman degradation is given at the top. The peak marked by a star has a mass increment of 16 corresponding to a fraction of the peptide in which the methionine residue was oxidized to the methionine sulfoxide. B: Fraction of the peptide containing several glutamylated components which are marked E1–E6 in addition to the unglutamylated form (E0). Stars are used as before. C: Mass spectrum of highly glycylation derivatives of the peptide labelled G2–G20 depending on the number of extra glycylation residues. The stars mark the methionine sulfoxide form as above. G0 is the non-glycylation peptide.

were further analyzed by HPLC. All peaks from the HPLC experiments were monitored by mass spectrometry and the major peaks were also characterized by automated Edman degradation. Fig. 2 gives an overview of the large number of variants encountered. The sequence of 24 residues established on a fraction containing essentially unmodified peptide (Fig. 2A) is given at the top of the figure. It fits a previous protein chemical study [11] and is additionally supported by a time course experiment with carboxypeptidase Y which proves that the final tyrosine residue is preceded by an alanine. Lack of further digestion is in line with the preceding cluster of acidic residues.

The major fractions of the α -peptide are the unmodified form (Fig. 2A) and a series of glycylation derivatives ranging from 2 to 20 extra glycylation residues (Fig. 2C). In this series components with 13 and 14 glycylation residues are particularly abundant. The mass spectrum of a HPLC fraction originating from a Mono Q fraction which eluted at high salt is shown in Fig. 2B. It contains in addition to the unmodified peptide a series of variants with 1–6 extra glutamyl residues. These glutamylated variants accounting for a minor fraction of the α -peptide were not detected in a previous analysis [11].

Because of technical difficulties with the amino acid sequencer and the limited amount of material available the position of the first glycylation residue in the sequences was not unambiguously identified. However, the available sequence data show that the modifications must lie in the last 10–14 residues, i.e. in the region where they have been precisely mapped in certain axonemal tubulins [10,12–16].

3.3. Immunological detection of glutamylated α - and β -tubulins

Immunological evidence for glutamylation of *Giardia* tubulins comes also from the reactivity of the glutamylation-specific tubulin antibody GT335 [25] in immunoblotting experi-

ments (Fig. 3). This monoclonal antibody detects both α - and β -tubulin.

3.4. Extensive acetylation of Lys-40 of cytoskeletal α -tubulin

The degree of acetylation of Lys-40 in α -tubulin is easily monitored when a CNBr digest is separated by SDS-PAGE, blotted onto a poly(vinylidene difluoride) membrane and the individual bands subjected to automated sequencing [1,17]. Fig. 4 shows the amino-terminal sequence of a large *Giardia* CNBr fragment and its alignment versus the corresponding

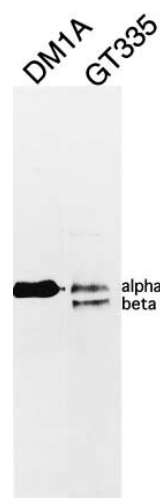


Fig. 3. Detection of glutamylated tubulin variants in immunoblotting with monoclonal antibody GT335. An aliquot of the cytoskeletal residue was used in SDS-PAGE using conditions which allow separation of α - and β -tubulin (see Section 2). Parallel immunoblots were processed with the general α -tubulin antibody DM1A, which stains the upper α -band, and with the glutamylation-specific antibody GT335, which detects both the α - and β -tubulin bands.



Fig. 4. Acetylation site of α -tubulin from *Giardia lamblia*. The upper line gives the amino-terminal sequence of the large CNBr fragment of *Giardia* (Giar) α -tubulin (M_r 14 000). At the fourth step only the phenylthiohydantoin of *N*- ϵ -acetyl-lysine was found (Acetyl-K in the sequence). The lower line gives the corresponding region and residue numbers of *Trypanosoma* (Tryp) α -tubulin predicted from the cDNA sequence [35]. Note the two amino acid exchanges at positions 44 and 45.

Trypanosoma sequence. At the fourth step corresponding to residue 40 only *N*-acetyl-lysine was detected. Thus Lys-40 seems completely acetylated.

4. Discussion

Tubulins from the stable microtubules retained in cytoskeletal preparations of *Giardia lamblia* were analyzed for post-translational modifications. Mass spectrometry and sequence analysis of the isolated carboxy-terminal peptides were used to monitor the tubulin specific modifications which locate to this region (for references see Section 1). The major variants of both β - and α -tubulin are the unmodified polypeptides and a series of polyglycylated derivatives reaching up to 15 and 20 extra residues respectively. Thus polyglycylation is the major tubulin-specific modification in *Giardia lamblia* (Figs. 1 and 2). Polyglutamylation, a second tubulin-specific modification, was only encountered as a minor fraction and with rather short side lengths. Up to two extra glutamyl residues were encountered in β -tubulin while in α -tubulin the maximal number observed was six. Some of the glutamylated β -tubulin variants were additionally glycylation by 1–12 glycylation residues. Modification of the same tubulin molecules by glycylation and glutamylation was also found in sperm axonemes of sea urchin and bull [12–14,16] where it also reflects minor species. In all these cases the number of extra glutamyl residues is low while glycylation can reach higher values (see also Fig. 1).

A previous study of *Giardia* α -tubulin concentrating on polyglycylation did not resolve the issue of a possible tubulin glutamylation [11]. To answer this question unambiguously we analyzed both α - and β -tubulin and monitored each peak of the anion exchange columns by HPLC and mass spectrometry. In addition we had access to the monoclonal antibody GT335, which detects glutamylated α -tubulins and some glutamylated β -tubulins. The antibody requires a glutamylation motif already provided by monoglutamylated α -tubulin and certain sequence elements around the glutamylation site. Thus its reactivity is independent of the degree of glutamylation beyond the first side chain residue [25]. Its reactivity on both α - and β -tubulin of *Giardia* cytoskeletons fully confirms the mass spectroscopic results on the carboxy-terminal peptides. In addition the immunoblot in Fig. 3 shows that in comparison to the general antibody for α -tubulin the glutamylated variants must be minor components as also seen in the mass spectroscopic results.

Giardia lamblia lacks the deetyrosination/tyrosination cycle. All of the many variants of the carboxy-terminal peptide of α -tubulin retain the terminal tyrosine indicative of the absence of a tubulin carboxypeptidase. In addition the terminal sequence DAY would not be accepted in deetyrosinated form

by tubulin tyrosine ligase. At least the mammalian enzyme adds the tyrosine only to a terminal glutamic acid and strongly prefers a penultimate glutamic acid [8]. Such a sequence, EEEY, is found in α -tubulin from *Trypanosoma*, which is known to have a deetyrosination/tyrosination cycle [17,28,29]. We also monitored the only tubulin modification outside the carboxy-terminal region. A previous immunoelectron microscopic study with a monoclonal antibody to acetylated α -tubulin [30] detected axonemal and cytoplasmic microtubules of *Giardia*. Our results confirm and quantitate these results. Automated sequencing of a corresponding CNBr fragment of α -tubulin shows that Lys-40 is fully acetylated (Fig. 4).

Ribosomal RNA and various protein phylogenies [18–21] suggest that *Giardia lamblia* reflects the oldest branch of the eukaryotic tree. Our results document that this ancient eukaryote has already nearly the entire complement of tubulin-specific posttranslational modifications established for other eukaryotes. *Giardia lamblia* shows polyglycylation and polyglutamylation of both α - and β -tubulin and the acetylation of α -tubulin. However, it lacks the deetyrosination/tyrosination cycle of α -tubulin, which is already present in *Trypanosoma* [17,28,29], a somewhat younger branch of the eukaryotic tree. Polyglycylation has so far only been observed in axonemal microtubules [10–16] but the lack of this modification in the flagellum of *Trypanosoma brucei* shows that polyglycylation is not a necessary requirement of axonemal structure [17]. Thus glutamylation is particularly interesting since it is present on microtubules from a wide variety of organisms including animals, ciliates, *Trypanosoma* and green algae [17,25,31–33] as well as *Giardia*. Its function seems to involve a regulation of the affinity of tubulin for certain microtubule-associated proteins [34].

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