Posttranslational modifications of trichomonad tubulins; identification of multiple glutamylation sites

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Abstract The α- and β-tubulins present in cytoskeletons of *Tritrichomonas mobilensis* are extensively glutamylated. Automated sequencing and mass spectrometry of the carboxyterminal peptides identifies 4 glutamylation sites in α- and 2 sites in β-tubulin. They are marked by asterisks in the terminal sequences GDE*E*E*E*DDG (α) and EGE*E*DEEAEA (β). This is the first report that tubulin glutamylation can occur at multiple sites. Although *T. mobilensis* has four flagellae the tubulins lack polyglycylation. Thus glycylation is not necessary for formation or function of axonemal microtubules. α-Tubulin is completely acetylated at lysine 40 and shows no tyrosine cycle. Peptide sequences establish two distinct β-tubulins.

Key words: Trichomonad; Polyglutamylation; Polyglycylation; Posttranslational modification; Tubulin

1. Introduction

Trichomonads, a group of primitive protists in the phylum *Parabasalia* [1], are interesting for two reasons. First, they include important human parasites, and second they belong to the earliest diverging eukaryotes known. Although not as old as *Giardia lamblia*, a member of the Diplomonads, Trichomonads are more ancient than the trypanosomes, which belong to the Kinetoplastida [2,3]. All these ancient eukaryotes have a pronounced microtubule dominated cytoskeleton including one (trypanosomes) or several flagella(e) which offers the opportunity of analyzing for a possible co-evolution of tubulin specific posttranslational modifications and the corresponding tubulin sequences.

Tubulin modifications fall into two groups. Acetylation of lysine 40 in some α-tubulins [4] and certain phosphorylation sites are more general modifications. All other modifications seem tubulin specific and involve the carboxyterminal 12 residues, usually a very acidic sequence. The terminal tyrosine of some α-tubulins participates in a detyrosination/tyrosination cycle based on a carboxypeptidase and the tubulin-tyrosine ligase, which was cloned from mammalian brain [5,6]. Additional removal of the penultimate glutamyl residue leads to an α-tubulin, which is no longer a substrate for the ligase [7]. In line with sequence changes the tyrosine cycle is not observed in some ciliates [8] and in *Giardia* [9] but is well documented for trypanosomes [10–12]. Polyglutamylation, based on isopeptide bond formation by the γ-carboxylate group of a particular glutamic acid residue, was first documented for brain α-tubulin [13] and subsequently found in the various brain β-tubulins [14–17]. It also occurs in axonemal microtubules such as sperm flagella of bull and sea urchin [18–21] and involves nearly all α- and β-tubulins in the subpellicular and flagellar microtubules of *Trypanosoma brucei* [12] but it is a minor modification in *Giardia* microtubules [22]. It is also found as minor modification in centriolar preparations of a green alga [23]. Finally α- and β-tubulins of cilia and flagella can be polyglycylated due to the formation of a side chain again connected via an isopeptide bond to a particular glutamic acid residue. Polyglycylation occurs in axonemal microtubules of the cilia of *Paramecium* [8], the sperm flagella of sea urchin [20,21] and bull [18,19] and the flagellated protist *Giardia* [9,22]. However, polyglycylation was not detected in the flagellum of *T. brucei* by mass spectrometry and radioactive labelling experiments [12].

To try to connect the information on posttranslational modifications in the oldest eukaryotes we used sequence analysis and mass spectrometry on the carboxyterminal peptides of tubulins from the trichomonad *Tritrichomonas mobilensis*. While our results confirm and extend previous immunological observations [24] we unexpectedly find that polyglutamylation involves multiple sites.

2. Materials and methods

2.1. Cells, cytoskeletal preparations and tubulin isolation

Trichomonas mobilensis was cultivated in Diamond's medium [25] supplemented with 10% heat inactivated horse serum at 37°C. Cytoskeletal residues of 2×10^9 cells were prepared as described [24], dissolved in SDS sample buffer, boiled and subjected to SDS-PAGE. Alternatively 6 M urea was included in the gel buffer to separate α- and β-tubulins. Tubulin bands stained by Coomassie brilliant blue were excised, briefly washed with water and kept frozen until use.

2.2. Protein chemical procedures

Tubulin present in stained gel fragments was electrophoretically concentrated by the agarose gel concentration system and digested with either trypsin or thermolysin [12,19,20,22]. These digests provided the carboxyterminal peptides of α-tubulin. The corresponding fragments of β-tubulin were obtained by CNBr treatment of gel fragments as described [18–20]. All peptide peaks from the HPLC profiles were characterized by mass spectrometry using KRATOS MALDI 3 or 4 time of flight mass spectrometers (Shimadzu, Duisburg, Germany) as described previously. Major peptide peaks were also analyzed by automated Edman degradation using instruments with online phenylthiohydantoin amino acid analysis.

For identification of lysine 40 of α-tubulin, gel fragments containing pure α-tubulin were subjected to in situ fragmentation by CNBr followed by SDS-PAGE and electrophoretic blot onto a poly(vinylidenedifluoride) membrane. A fragment with an apparent M_r of 4000 was subjected to automated sequencing, which resolves the phenylthiohydantoin derivatives of lysine and *N*-ε-acetyllysine [12,22].

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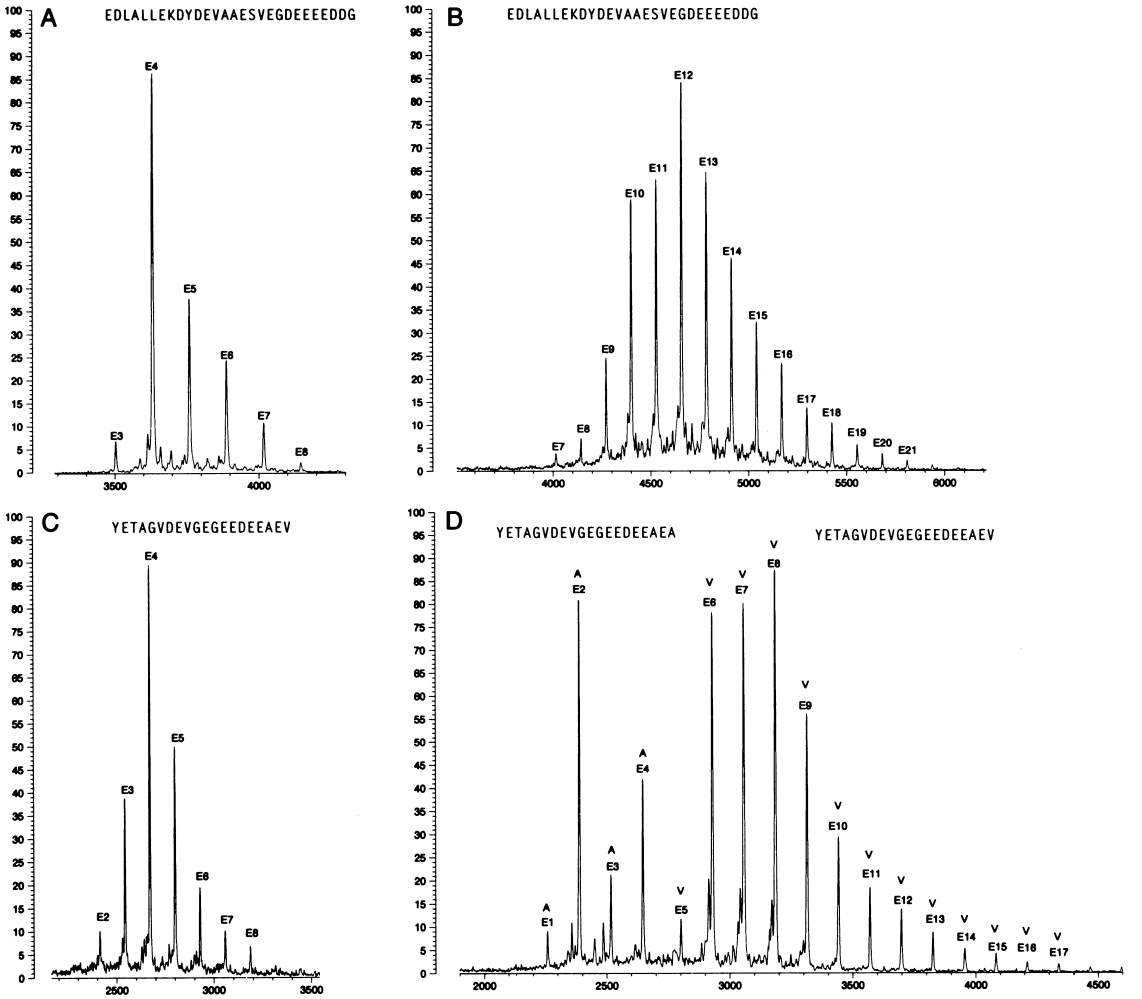

Fig. 1. Mass spectra of variants of the carboxyterminal peptides of α- and β-tubulin from *Tritrichomonas mobilensis*. A,B: MALDI mass spectra of two fractions containing low (A) and highly glutamylated (B) variants of the α-tubulin peptide. The amino acid sequence of the peptide generated by trypsin is shown at the top. The glutamylated species are marked as E3–E21 according to the number of extra glutamyl residues. C,D: MALDI mass spectra of two fractions of the glutamylated variants of the carboxyterminal β-tubulin peptides generated by CNBr. The sequences of the peptides are shown at the top. The glutamylated species are marked as E1–E17 according to the number of extra glutamyl residues. A and V in panel D mark the two distinct β-tubulins carrying either a carboxyterminal alanine (A) or valine (V) as indicated in the two sequences at the top. Peptides in panel C have a carboxyterminal valine (see sequence). Only four of the numerous HPLC fractions are shown. For details see Section 3.

3. Results

Cytoskeletal preparations of *Tritrichomonas* obtained by Triton X-100 extraction in the presence of protease inhibitors are dominated in SDS-PAGE by the tubulin band [24]. Inclusion of 6 M urea in the buffer of the running gel resulted in separation of α- and β-tubulin. Stained gel pieces were used to isolate and characterize the carboxyterminal fragments of both tubulins. All digests were first chromatographed on a small Mono Q column to isolate the acidic carboxyterminal fragments. The later emerging peaks were subjected to further separation by reverse phase HPLC columns. All peak fractions from the HPLC columns were monitored by mass spectrometry (Fig. 1) and major peaks were characterized by automated Edman degradation (Figs. 2 and 3).

The carboxyterminal sequence of *Tritrichomonas* α-tubulin derived by cDNA analysis is KEDLALLEKDYDEVAAESVEGDE*E*E*E*DDGQQ. The carboxyterminal fragments derived by trypsin and thermolysin cleavage were found to start with the sequences EDLALL... and LEKDYD..., respectively. They follow the predicted sequence except that at the glutamic acid E positions which are marked by an asterisk no phenylthiohydantoin derivative was obtained (see Fig. 2 for the thermolytic fragment). Since the sequence continued normally for three further steps the four glutamic acid residues marked by an asterisk must carry a glutamyl side chain as indicated by the mass spectrometric data (see Fig. 1 for the tryptic peptide). In none of six independent Edman degradations did we find the two terminal glutamine residues predicted by the cDNA sequence. We therefore assume that they were lost by proteolysis either in the living cells or during the preparation of the cytoskeletal residues. The mass spectrometric results show that α-tubulin is extensively glutamylated with the total number of extra glutamyl residues ranging from 2–20. The main components have 3–12 extra glutamyl residues (Fig. 1), which by Edman degradation arise at four

distinct glutamylation sites. The non-glutamylated peptide was present only in trace amounts and no glycinated α-tubulin peptide was detected.

Characterization of the carboxyterminal peptides of β-tubulin from a tryptic digest showed extensive glutamylation on a peptide starting with the sequence SNMTDLIQEYEMYETAGVDEVGEG. This sequence corresponds, with two amino acid replacements (underlined) to the carboxyterminal tryptic peptide from *Trichomonas vaginalis* [26]. To obtain a shorter fragment, gel purified β-tubulin was treated with CNBr and the digest processed as above. Several major peaks from the HPLC column characterized by Edman degradation yielded the sequence YETAGVDEVGEGE*E*DEEAE followed in one case by A and in the other by V. This result established the presence of at least two distinct β-tubulin isotypes in *Tritrichomonas mobilensis* in agreement with previous DNA sequences of *Trichomonas vaginalis* [26]. At the positions marked by an asterisk, which are glutamic acid residues in *Trichomonas vaginalis*, no phenylthiohydantoin derivative was obtained in Edman degradation (Fig. 3). Since the sequence continued normally for 6 further steps the two glutamic acid residues marked by an asterisk must carry a glutamyl side chain as indicated by the mass spectrometric results (Fig. 1). The two isotypes ending in valine and alanine respectively (see above) account for about 60 and 35% of the carboxyterminal peptides. The mass spectrometric results show that β-tubulin is extensively glutamylated with the total number of extra glutamyl residues ranging from 2 to about 17. The main components have 2–9 extra glutamyl residues, which by Edman degradation arise at two distinct glutamylation sites. No peptide from non-glutamylated or glycinated β-tubulin was detected. A minor, heavily glutamylated component, accounting for less than 5% of the material, lacks the last residue of the two major species. It could only be characterized by mass spectrometry. Two further species, which were only detected in trace amounts, are compatible with sequences in which the major species ending in alanine (see above) has one and two additional alanines. The trace component ending with three

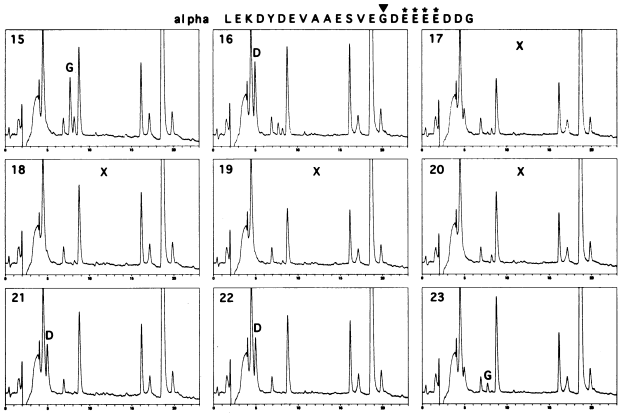

Fig. 2. Identification of four glutamylation sites in *Tritrichomonas* α-tubulin. The sequence of the heavily glutamylated carboxyterminal peptide obtained by thermolysin digestion is shown at the top. The series of consecutive Edman degradations documented starts with G at step 15 (marked by an arrowhead in the sequence). The amino acid residue identified in each step is given in bold type. Note that no phenylthiohydantoin derivative is found at steps 17, 18, 19 and 20 (indicated by X) which involve the four glutamic acid residues marked by an asterisk in the sequence. In the following steps 21–23 the sequence continues normally.

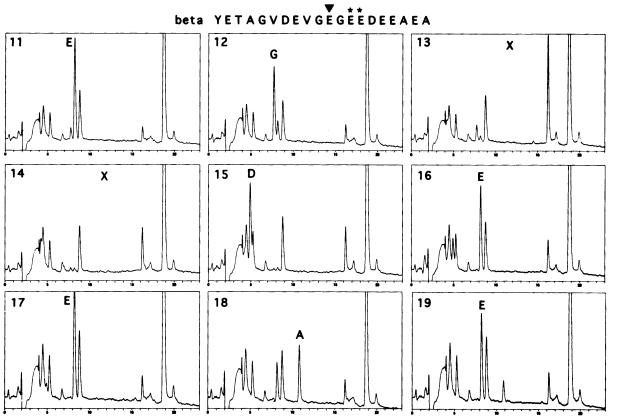

Fig. 3. Identification of two glutamylation sites in *Trichomonas* β-tubulin. The sequence of the heavily glutamylated carboxyterminal CNBr fragment is shown at the top. The series of consecutive Edman degradations documented starts with E at step 11 (marked by an arrowhead in the sequence). The amino acid residue identified in each step is given in bold type. Note that no phenylthiohydantoin derivative is found at steps 13 and 14 (indicated by X), which involve the two glutamic acid residues marked by an asterisk in the sequence. In the following steps 15–20 (only 15–19 are shown) the sequence continues normally.

alanines fits the carboxyterminal end of β-tubulin from *Trichomonas vaginalis* [26]. The existing minor species indicate a proteolytic trimming either in the living cell or during the preparation of the cytoskeletons.

To quantitate the degree of acetylation of lysine 40 in α-tubulin a CNBr digest was separated by SDS-PAGE, using a highly resolving Tricine-SDS gel. The fragments were blotted onto a membrane and characterized by automated sequencing. While in most α-tubulins lysine 40 lies in a large fragment starting with proline 37, the presence of an additional methionine at position 75 moved the corresponding CNBr fragment of *Tritrichomonas mobilensis* to the 4000 molecular weight range. Its sequence PSDKTIGVEDDAF gave in the fourth step only the phenylthiohydantoin derivative of *N*-acetyllysine (underlined K in the sequence). Thus the α-tubulin of stable microtubules seems extensively acetylated at lysine 40.

4. Discussion

We have quantitated the posttranslational modifications present on the stable microtubules of *Tritrichomonas mobilensis*, a member of the trichomonads, by protein sequence analysis and mass spectrometry and compared the results with data on *Giardia* [9,22] and *Trypanosoma brucei* [12]. In all these ancient eukaryotes the α-tubulin is completely acetylated at lysine 40. Thus tubulin acetylation seems a very old and widespread modification, which, however, was lost in the fungi due to the sequence changes which include lysine 40 [3]. The functional role of tubulin acetylation, if any, is unclear since it can be genetically abolished without apparent consequences in *Tetrahymena* [27]. Due to sequence changes the tyrosine cycle of α-tubulin is not possible in *Giardia* [9], the oldest eukaryote, and trichomonads, which are clearly older than the trypanosomes for which the cycle is well established [10–12]. Polyglycylation of both α- and β-tubulin, thought to be a marker of axonemal microtubules [8], is already observed in *Giardia* [9] but was lost on the branches leading to trichomonads (see Section 3) and trypanosomes [12]. Since these

parasites have flagellae with a normal axonemal ultrastructure polyglycylation seems not connected with formation or function of axonemes, at least in these two organisms. The presence of polyglutamylation in *Giardia* [22], trypanosomes [12] and trichomonads (see Section 3) supports the notion that this posttranslational modification is very old and very widely spread. While it is a minor modification in *Giardia* the α- and β-tubulins of the stable microtubules in both trypanosomes and trichomonads seem completely glutamylated. Although the detailed function of this modification still needs to be established some results indicate that the oligoglutamyl side chains regulate in a length dependent manner the binding of microtubule associated proteins [28].

Polyglutamylation was always thought to involve only one particular glutamic acid residue in the carboxyterminal sequences of brain α- and β-tubulin [13–18]. Our results on *Tritrichomonas* are the first report on the presence of multiple sites. Extensive Edman degradation studies and mass spectrometry led to the identification of 4 sites in α- and 2 sites in β-tubulin (Figs. 2 and 3). This situation is somewhat reminiscent of recent results on polyglycylation. Isolated axonemal microtubules from *Paramecium* cilia consist of α- and β-tubulins which are completely glycylated. They carry between 1 and 34 extra glycyl units thought to reflect single chains extending from one site only [8]. However, in sea urchin sperm axonemes glycylation seems restricted to β-tubules where it involves only some 45% of the tubulins present. Extensive Edman degradation patterns established at least two independent sites for α- and three sites for β-tubulin [20]. Interestingly a recent mass spectrometric study using collision induced dissociation and postsource decay on a hexaglycylated *Paramecium* β-tubulin peptide established four glycylation sites which involve the last four glutamic acid residues [29].

Currently we do not know whether the presence of multiple glutamylation sites in *Tritrichomonas* is related to changes in the carboxyterminal tubulin sequences. It will be interesting to see whether multiple glutamylation sites also occur in other tubulins. They do not seem to be present in the major α- and β-brain tubulins [13–17] but have to be considered in cases of extremely high glutamylation levels [23]. In any approach to understand the possible function of tubulin glutamylation [28] it is important to distinguish between a single polyglutamyl sidechain and several shorter oligoglutamyl sidechains.

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