β tubulin of bull sperm is polyglycylated

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Abstract Carboxy-terminal fragments of α and β tubulin from bull sperm were isolated and characterized by automated sequencing and mass spectrometry. About 60% of sperm α tubulin is polyglycylated. The lateral chain, which can reach 13 residues in length, is covalently attached via an isopeptide bond. The fully detyrosinated sperm α tubulin lacks polyglycylation. Thus mammalian sperm microtubules differ from the ciliary axonemal microtubules of the protozoan *Paramecium* for which others have documented a complete polyglycylation of both α and β tubulin.

Key words: Flagellum; Cilia; Polyglutamylation; Polyglycylation; Sperm; Tubulin

1. Introduction

Microtubules are involved in cell division, directed intracellular transport and the movement of cilia and flagella. In addition, microtubules influence the dynamic organisation of cellular morphology [1]. The αβ heterodimer, the structural unit of microtubules, can display high molecular heterogeneity due to the presence of different α and β isotypes, which are encoded by distinct genes, and a set of post-translational modifications, which seem to vary with the cell type. Tubulin from mammalian brain shows a particularly impressive complexity. With the exception of Lys-40 in α tubulin [2] all currently known post-translational modifications locate to the very acidic carboxy-terminal region of tubulin, which is thought to be involved in the binding of microtubule associated proteins [3,4].

The carboxy-terminal tyrosine of α tubulin is removed by a specific carboxypeptidase. Tubulin-tyrosine ligase can add a further tyrosine provided that the penultimate glutamic acid residue has not been previously removed [5–7]. The tubulin-tyrosine ligase is well characterized and acts on the carboxy-terminal tetradecapeptide of detyrosinated α tubulin, albeit at low rates [7,8]. Both α and β tubulins from adult brain display polyglutamylation which occurs at specific glutamyl residues. The length of the lateral chains can reach 8 residues [9], although the majority may be only 2–3 residues long and a pool of non-glutamylated tubulin is still present [10–14]. Finally, Ser-444 and Tyr-437 of brain βIII tubulin are partially phosphorylated [10,15]. In contrast to brain tubulin the microtubules of avian erythrocytes provide an unusually homogeneous preparation based on one α (α1) and one β (β6) chain. The completely detyrosinated α tubulin has a full complement of the penultimate glutamic acid and lacks acetylation of Lys-40. Thus the only modification which we observed is a substoichiometric phosphorylation of Ser-441 in β6 [16].

Given the minimal modifications of tubulin by post-translational modifications in the marginal band microtubules of avian erythrocytes, we turned to another specialized microtubular system, the sperm flagella. Here we show by sequence analysis and mass spectrometry that more than half of the β tubulin from bull spermatozoa is modified by polyglycylation, a modification recently described by Redeker et al. [17] for axonemal microtubules of the protozoan *Paramecium*. While in the ciliate both α and β tubulins are polyglycylated, in mammalian sperm this modification involves only the β subunit.

2. Material and methods

2.1. Tubulin purification

Bull sperm, which had been kept at −80°C, was a gift from Dr. K.-H. Scheit. Tubulin was purified as described [18]. Washed bull spermatzoa were briefly sonicated to separate sperm heads and tails. Tails were purified by repeated differential centrifugation. Isolated tails were resuspended and extensively sonicated in MES assembly buffer (100 mM MES-KOH, pH 6.8, 2 mM $MgCl_2$, 0.5 mM DTT, 0.2 mM PMSF, 1 mM GTP). Extracts were cleared by centrifugation (100,000 × g; 30 min at 4°C). The tubulin containing supernatant was applied to a MonoQ HR 5/5 column (Pharmacia) equilibrated in 10 mM Na-phosphate, pH 7.0, and protein was eluted with a linear gradient from 0 to 0.6 M NaCl in this buffer. Tubulin containing fractions were pooled and dialyzed against MES assembly buffer. α and β tubulin were separated by preparative SDS-urea-PAGE gels as described [13]. Gels were briefly stained with Coomassie brilliant blue. The bands corresponding to α and β tubulin were cut out.

2.2. Protein cleavage procedures

2.2.1. Gas-phase CNBr cleavage. Gel pieces containing α or β tubulin were applied to a second concentrating gel and subsequently blotted on to PVDF. The membrane was exposed to the vapour of a CNBr solution (5 mg/ml in 70% formic acid) in a closed reaction tube. After 16 h at room temperature the membrane was washed twice with 100 μl 80% TFA and twice with 70% acetonitrile. Wash fractions were combined, dried, redissolved in 10 μl of 0.1% TFA and directly analyzed by mass spectrometry (see below).

2.2.2. CNBr cleavage in acrylamide gels. Gel pieces from preparative SDS-PAGE were treated at room temperature with 40% *n*-propanol and then with 50% acetonitrile. Extracted gel pieces were air-dried for 30 min. Protein cleavage was for 16 h at room temperature using 500 μl of 5 mg/ml CNBr in 70% formic acid. The supernatant was removed and the gel pieces were washed for 30 min at 37°C twice with 300 μl 70% formic acid and twice with 300 μl of 80% acetonitrile, 0.05% TFA. The combined supernatant and wash fractions were dried. Peptides were redissolved in 80 μl of 80% acetonitrile, 0.05% TFA.

2.2.3. Endoproteinase LysC cleavage. Gel pieces containing α and β tubulin were applied to a concentrating gel. The highly concentrated protein band was treated with endoproteinase LysC (3 μg/ml for 16 h, 37°C).

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Abbreviations: HPLC, high-performance liquid chromatography; MALDI, matrix assisted laser desorption ionization; PVDF, polyvinylidenedifluoride; TFA, trifluoroacetic acid; TOF, time of flight.

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2.3. Chromatographic procedures

Buffer exchange of samples from cleavage procedures was done on a SMART Fast Desalting column (Pharmacia) equilibrated in buffer A (20 mM Na-phosphate, pH 7.0). Peptides were separated on a SMART MonoQ column equilibrated in buffer A with a 2.4 ml linear gradient (0 to 0.5 M NaCl in buffer A) at a flow rate of 100 μl/min. Elution profiles were monitored at 214 nm and peaks were collected manually. Peak fractions were analyzed by amino-terminal sequencing (see below). Fractions containing the carboxy-terminal fragments were subjected to reverse-phase HPLC on a Vydac 218 Tp52 column. Peptides were eluted with a 9 ml linear gradient from 10 to 90% solvent B at a flow rate of 100 μl/min. Solvent A 0.1% TFA, solvent B 70% acetonitrile in 0.08% TFA. Peak fractions were characterized by amino-terminal sequencing and by mass spectrometry (see below).

2.4. Amino-terminal sequencing

Peptides were sequenced by automated Edman degradation using an Applied Biosystems gas-phase sequenator (model A470) or a Knauer sequenator (model 810). Both instruments were equipped with an on-line PTH-amino acid analyser.

2.5. Mass spectrometry

Mass spectra were recorded with a Bruker REFLEX-time-of-flight mass spectrometer using sinapinic acid dissolved in 40% acetonitrile, 0.06% TFA. Samples were prepared as described in detail elsewhere [9]. Spectra of negative ions were recorded in the reflected mode. The instrument was calibrated with bovine insulin and a mixture of standard peptides [9].

3. Results

3.1. Glutamylation of mammalian sperm α tubulin

αβ tubulin was concentrated in an agarose gel and digested with endoproteinase LysC. The resulting digest was chromatographed on a MonoQ column to isolate the acidic carboxy-terminal peptides. All peak fractions eluting from the column were characterized by sequence analysis. Fractions 22 and 23 yielded the sequence DYEEVEVDXVEAE... where X is a not yet identified residue. This partial sequence is in agreement with published sequences for testis specific α tubulin from mouse (Genbank accession no. M13443) and human (Genbank accession no. L11645) (Fig. 1). The fractions were pooled and further analyzed by reverse-phase HPLC. The peptide eluting in fraction 8 was analyzed by MALDI-TOF mass spectrometry (Fig. 2). The major peak at a mass-to-charge ratio of 2084 corresponds to the detyrosinated carboxy-terminal peptide ^{431}DYEEVGVDSVEAEAEGEE^{449} (see Fig. 1). The smaller peak at 2213 differs by an increment of 129 which corresponds to an additional glutamyl residue. The small peak at 2344 probably corresponds to a peptide with two added glutamyl residues. Given the large literature on glutamylation of brain tubulin [10–14], we conclude that α tubulin from bovine sperm is also glutamylated, albeit at a lower degree.

We did not detect any tyrosinated α peptides. This is in agreement with the finding that sperm α tubulin hardly reacts with antibody YL 1/2 directed against tyrosinated tubulin. It is, however, stongly recognized by antibody ID5, which is specific for detyrosinated α tubulin (data not shown; for antibody characteristics see [19]). In a sample prepared with α-cyano-4 hydroxycinnamic acid as matrix an additional small peak at 1995 was detected. This probably represents a peptide lacking not only the tyrosine but also the penultimate glutamic acid residue (Glu-449). This partial loss of the penultimate residue has been well documented in brain α tubulin [5,8,13].

3.2. Glycylation of mammalian sperm β tubulin

Probably because of its relatively large size (residues 392–445) we did not find the expected carboxy-terminal fragment of β tubulin in the endoproteinase LysC digest. We therefore

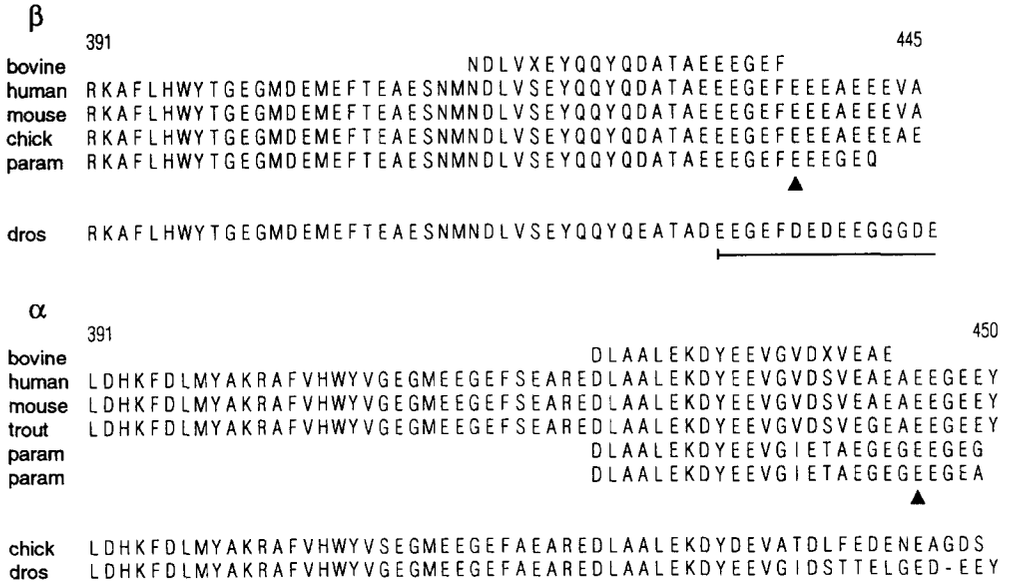

Fig. 1. Sequence alignments of the carboxy-terminal regions of α and β tubulins from animal sperm and the ciliate *Paramecium* (param). Swiss Prot Accession numbers (if not indicated otherwise) for α and β respectively are: human L11645 (GenBank), P05217; mouse P05214,[20]; chick P08070, P09206; *Paramecium* [17], P33188; *Drosophila* P06604, P08840; trout P18288. The position of the lateral polyglycine chains in both α tubulins and the β chain of axonemal microtubules from *Paramecium* (arrowheads in α and β) are from Redeker et al. [17]. The alignment of the carboxy-terminal peptides of α and β tubulin from bull sperm (this study) are shown at the top. Although these peptides were not fully sequenced they could be unambigously characterized by mass spectrometry (see section 3). X indicates a residue not yet identified by sequence analysis. Note the high degree of sequence conservation, except for the last 5 residues, in the β tubulins and a lower degree of conservation in the α tubulin. Note also that polyglycylation in bull sperm is restricted to β tubulin (Fig. 3) while α tubulin is subject to detyrosination and glutamylation (Fig. 2). The bar under the carboxyl end of *Drosophila* (dros) β tubulin marks the extent of a 15 residue deletion. This mutant β tubulin forms normal microtubules but is unable to organize the microtubule-based spermatogenic suprastructures [27].

performed gas-phase CNBr cleavage of β tubulin blotted on to PVDF. The fragment mixture eluted from the membrane was directly analyzed by MALDI-TOF mass spectrometry (Fig. 3A). The major peak at a mass-to-charge ratio (m/z) of 3482 is in perfect agreement with the calculated values for the carboxy-terminal CNBr fragments of mouse [20] and human [21] β-tubulin (Fig. 1). Additionally, we found a group of 5 peaks between 3939 and 4167. They differ by multiple units of 57 from the unmodified peak at 3482. Thus, for example the mass of 3939 arises as 3482+(8 × 57). This type of modification, i.e. polyglycylation, has recently been described by Redeker et al. [17] for axonemal tubulin from *Paramecium*. Thus the additional peaks in Fig. 3A seem to represent the carboxy-terminal β-peptide ^{416}NDLVSEYQQYQDATAEEEEFEEEAEEEVA^{445} carrying 8–12 glycyl residues as a lateral side chain. Using sperm axonemes from the sea urchin *Psammechinus miliaris*, kindly provided by Dr. E.M. Mandelkow, we observed a similar glycylation pattern for β tubulin, while α tubulin lacked this modification.

To isolate larger amounts of carboxy-terminal peptides of bull sperm β tubulin for further characterization, we performed CNBr cleavage in the gel pieces (see section 2). Acidic peptides were again purified on a MonoQ column. Peak fractions 23–26 all yielded the sequence NDLVSEYQQYQDATAEEEGEF..., corresponding to β416–445 (see Fig. 1). Combined fractions 23/24 and 25/26 were subjected to reverse-phase HPLC and the peaks obtained were characterized by amino acid composition, amino-terminal sequencing and mass spectrometry. MonoQ fractions 23/24 contained the glycylated carboxy-terminal β-peptides (Fig. 3C,D), while fractions 25/26 contained the unmodified carboxy-terminal β-peptide (Fig. 3B). Thus, the glycylated peptides show a less acidic behaviour on MonoQ than does the unmodified peptide. Interestingly an unusual behaviour is also observed on the reverse-phase column where the unmodified peptide elutes later than the glycylated peptides. The peptides carrying 7–10 glycyl units elute in fraction 11 (Fig. 3C), while those carrying 9–13 glycyl units elute already in fraction 9 (Fig. 3D). Fig. 3A shows that a significant amount of sperm β tubulin is not modified. We estimate from the peak heights that not more than 60% of the sperm β tubulin is glycylated.

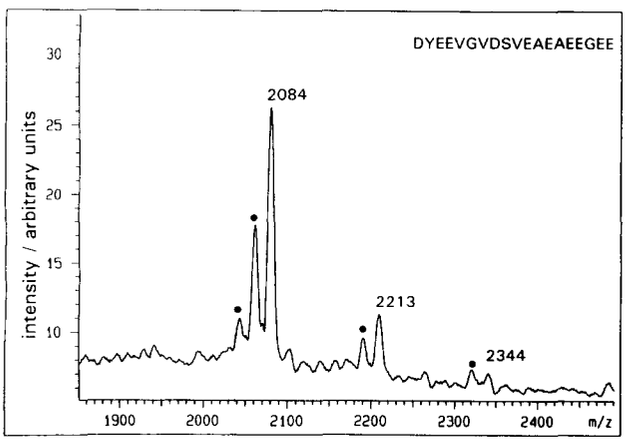

Fig. 2. Mass spectrum of the carboxy-terminal peptides of sperm α tubulin generated with endoproteinase LysC. The sequence of the unmodified α-peptide at 2084 is indicated. Metastable peaks generated by the elimination of water are marked with a dot. The minor peaks at 2213 and 2344 correspond to the glutamylated α-peptide carrying 1 and 2 additional glutamyl residues.

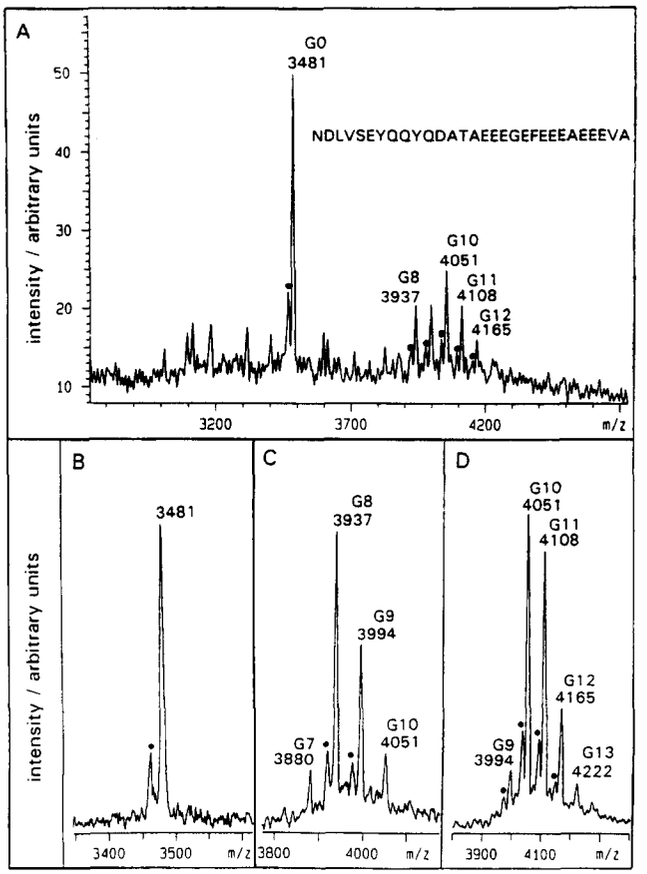

Fig 3. Mass spectra of the carboxy-terminal peptides of sperm β tubulin generated by CNBr cleavage. Panel A shows the spectrum of β tubulin peptides eluted from PVDF after gas-phase CNBr cleavage. The sequence of the unmodified peptide β416–445 at 3481 (G0) is indicated. The glycylated peptides are marked as G8–G12 according to the number of additional glycyl residues. Panels B–D show spectra originating from CNBr cleavage in solution. Panel B gives the spectrum of the peptide in MonoQ fractions 25/26. Panels C and D show the peptides in MonoQ fractions 23/24 after reverse-phase HPLC purification. Panel C gives the moderately modified peptides from HPLC fraction 11 carrying 7–10 glycyl units. Panel D presents the higher glycylated β-peptides from HPLC fraction 9 bearing 9–13 glycyl units. Dots are used as in Fig. 2. All indicated average masses were determined from several independent measurements. The differences beween the experimental average mass-to-charge ratios (m/z) and the calculated values range from 0.1 to 1.8 Da, consistent with the accuracy of MALDI-TOF mass spectrometry

4. Discussion

Using protein sequencing and mass spectrometry we have identified the post-translational modifications in the carboxy-terminal region of the stable microtubules present in bull spermatozoa. The β tubulin carries a lateral polyglycyl chain, a modification recently documented for both α and β tubulin in the axonemal microtubules of the ciliate *Paramecium* [17]. However, unlike in *Paramecium* the α tubulin of spermatozoa recovered in this study is not polyglycylated and shows instead a short lateral chain of 1–2 glutamic acid residues. This is consistent with immunological data indicating a glutamylation

Table 1
Post-translational modifications in α and β tubulin

Modification	Isotype	Sequence	Source	Reference
Acetylation on Lys-40	α1	QMPSD**K**TIGGGDD		[2]
Tyrosination/detyrosination of Tyr-451	α1	SVEGEGEEEGEE(**Y**)	Brain	[6,7]
Removal of Glu-450	α1	SVEGEGEEEGE	Brain	[5,13]
Phosphorylation on Ser-444	βIII	YEDDEEE**S**EAQGPK	Brain	[10]
Phosphorylation on Tyr-437	βIII	**Y**EDDEEESEAQGPK	Brain	[15]
Phosporylation on Ser-441	βVI	EYEEVEA**S**PEKET	Erythr.	[16]
Polyglutamylation on Glu-445	α1	VDSVEGEG**E**EEGEE(Y)	Brain	[11–13]
		VDSVEGEG**E**EEGE		
Polyglutamylation	βα	VDSVEAEAEE-GEE(Y)	Sperm	This work
Polyglutamylation on Glu-438	βIII	Y**E**DDEEESEAQGPK	Brain	[10]
Polyglutamylation on Glu-435	βII	DATADEQG**E**FEEEEGEDEA	Brain	[12,13]
Polyglutamylation on Glu-441	βI	EEDFGEEA**E**EEA	Brain	[14]
Polyglutamylation on Glu-440	βIVa	DATAEEEG**E**FEEEAEEEVA	Brain	[14]
Polyglycylation on Glu-445	Pα1	DYEEVGIETAEGEG**E**EGEG	Axoneme	[17]
	Pα2	DYEEVGIETAEGEG**E**EGEA		
Polyglycylation on Glu-437	pβ	DATAEEEGEF**E**EEGEQ	Axoneme	[16]
Polyglycylation	BβIVb	DATAEEEGEFEEEAEEEVA	Sperm	This work

Except for the acetylation of Lys-40 of α tubulin all other modifications involve the acidic carboxy-terminal regions. Tyrosination, polyglutamylation and polyglycylation seem reactions restricted to tubulins. Isotypes refer to vertebrate tubulins. *Paramecium* tubulins are marked P. Bull sperm tubulin is marked B. Note that tyrosination of previously detyrosinated α tubulin can also occur on some additional α types [8]. Tubulin isotypes are grouped into classes according to Sullivan [28]. Residues carrying modifications are printed in bold. The exact position of the particular glutamic acid residue modified by the lateral polyglycine chain in bull sperm β tubulin has not been determined. In analogy to *Paramecium* β tubulin it could be positioned at Glu-437.

of tubulin in sperm [22,23]. Glutamylation has also been documented for all α and β isotypes in a subpopulation of microtubules of adult mammalian brain [10–14]. A further difference between *Paramecium* and sperm microtubules is the observation that β tubulin is only partially polyglycylated in spermatozoa (maximally 60%, see section 3), while in the ciliate all tubulins of the axonemes seem fully polyglycylated [17]. With up to 34 glycyl residues the lateral chain is much longer than in bull sperm β tubulin where we detected only up to 13 residues.

With the exception of the acetylation of Lys-40 of α tubulin, all currently known post-translational modifications of tubulins locate to the very acidic carboxy-terminal region and have been positioned within the last 15 residues (Table 1). Several of these modifications (tyrosination of α tubulin, polyglutamylation and polyglycylation) seem reactions specific for tubulins as substrates. It is thought that the acidic region of tubulin, which seems to be involved in binding of some microtubule associated proteins [3,4,24,25], can be modulated by the modifications. Thus it is interesting to note that the single β2 tubulin of *Drosophila* spermatozoa, a mutant protein which lacks the carboxy-terminal 15 residues, can still form microtubules. However, this β tubulin mutant is unable to organize the microtubule-based spermatogenic superstructures [26,27]. Currently the presence of a polyglycyl chain in *Drosophila* spermatozoa can only be inferred from the high sequence conservation of sperm β tubulins (Fig. 1) and the documented presence of the side chain in bull sperm and *Paramecium* axonemes. However, future experiments based on two-dimensional gels of testes from *Drosophila* or a more convenient arthropod could be used to verify the substitution directly. In this case the analysis of *Drosophila* β2 tubulin can be refined by shorter deletions and point mutations. Already the current results indicate that the lateral polyglycyl side chain may have a direct function in proper axoneme assembly.

A potentially interesting aspect of polyglycylated tubulin is seen in the behaviour of the modified carboxy-terminal peptides during anion-exchange chromatography. The higher modified peptides elute earlier from DEAE [17] and MonoQ (this study). The lateral side chain seems to decrease the acidity, possibly by hydrogen bonds between some glutamic acid residues of the main chain and glycyl peptide bonds in the lateral chain.

Thus far the enzymes responsible for polyglutamylation and polyglycylation of tubulins have not been purified. However, in the case of tubulin-tyrosine ligase the detyrosinated tetradecapeptide from the carboxyl end of α tubulin can serve as the substrate, albeit at a 50 fold lower reaction rate than αβ tubulin [8]. It remains to be seen whether similar synthetic peptides can be used to detect the other modifying enzymes and to purify them from suitable tissues.

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