Axonemal tubulin polyglycylation probed with two monoclonal antibodies: widespread evolutionary distribution, appearance during spermatozoan maturation and possible function in motility

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SUMMARY

Two monoclonal antibodies, AXO 49 and TAP 952, probed with carboxy-terminal peptides from Paramecium axonemal tubulin and with polyglycylated synthetic peptides, are found to recognize differently tubulin polyglycylation, the most recently identified posttranslational modification discovered in Paramecium axonemal tubulin. With these antibodies, we show that tubulin polyglycylation is widely distributed in organisms ranging from ciliated protozoa to mammals; it arose early in the course of evolution, but seems to be absent in primitive protozoa such as the Euglenozoa. Tubulin polyglycylation is the last posttranslational modification which takes place in the course of Drosophila spermatogenesis and its occurrence corresponds to the end of spermatozoan maturation. An involvement of polyglycylated tubulin in axoneme motility is suggested since AXO 49 and TAP 952 specifically inhibit the reactivated motility of sea urchin spermatozoa.

Key words: Polyglycylation, Posttranslational modification, Tubulin, Monoclonal Antibody, Flagella, Spermatozoa, Spermatogenesis, Spermatozoan motility

INTRODUCTION

In eukaryotes, a panel of posttranslational modifications (PTMs) leads to the differentiation of a family of tubulin isoforms at the molecular level, and of various classes of microtubules at the cellular level. Although no direct biological function has been found for these PTMs to date, in most cases posttranslationally modified tubulin is found to accumulate in relatively stable microtubules. This is true at least for acetylation, detyrosylation (for reviews, see Greer and Rosenbaum, 1989; Bulinski and Gundersen, 1991; Gelfand and Bershadsky, 1991), phosphorylation (Gard and Kirschner, 1985) and β2-tubulin formation (Paturle-Lafanèche et al., 1994). As for polyglutamylation, while the polymer has also been suggested to be the preferred substrate (Audebert et al., 1993), polyglutamylated tubulin is detected not only in stable microtubules, but in labile ones too and within the soluble pool (Audebert et al., 1993; Bré et al., 1994).

The presence of a putative new PTM had first been hypothesized in axonemal tubulin thanks to the specific reactivity pattern of a polyclonal anti-tubulin antibody (Adoutte et al., 1991). This led us to raise a library of monoclonal antibodies against Paramecium axonemal tubulin which were tested by immunoblotting on peptide maps of axonemal tubulin and on ciliate tubulin fusion peptides (Callen et al., 1994; Levilliers et al., 1995), and by immunofluorescence on Paramecium cells (Fleury et al., 1995; Levilliers et al., 1995); accordingly, two of them, AXO 49 and TAP 952, were suggested to be directed against PTMs. Similarly as for most tubulin PTMs, the epitope recognized by AXO 49 is removed after subtilisin treatment, and therefore located near the carboxy-terminal end of α- and β-tubulin (Levilliers et al., 1995). The delayed detection of this epitope with respect to morphogenesis of microtubular structures during cell division in Paramecium cells (Fleury et al., 1995; Levilliers et al., 1995), and in Drosophila spermatozoa (Bressac et al., 1995) provided another argument for a PTM as target of AXO 49.

Therefore we proceeded to the chemical analysis of the carboxy-terminal part of Paramecium axonemal tubulin, and discovered a novel PTM, polyglycylation, consisting of the addition of up to 34 glycyl units to the γ carboxyl groups of
MATERIALS AND METHODS

In the present study, we first demonstrate, by dot-blot analysis of *Paramecium* axonemal tubulin peptides and of synthetic peptides, that AXO 49 and TAP 952 recognize polyglycylated tubulin. Therefore we report here the original synthesis of a class of ‘difficult sequences’, the branched polyglycylated tubulin peptides, which were designed to this aim. Then, through the use of the wide panel of *Paramecium* axonemal tubulin peptides tested with the two antibodies, we show that their respective affinities for low and high levels of modification differ, AXO 49 being more selective than TAP 952 towards higher degrees of glycylation.

Since AXO 49 and TAP 952 were proved to be complementary tools for the detection of tubulin polyglycylation, they were used together to compare the kinetics of appearance of polyglycylation to those of other PTMs during spermatogenesis in *Drosophila*. The late detection of both AXO 49 and TAP 952 epitopes contrasts with the early occurrence of the other PTMs, which leads us to infer that tubulin polyglycylation is a marker of the end of maturation in spermatozoa.

The fact that polyglycylation appears to be developmentally regulated during flagellar maturation raises the question of its role and hence of its conservation in the axoneme in the course of evolution. We therefore used our antibodies to probe the evolutionary distribution of polyglycylation in eukaryotic axonemes. Both antibodies recognize axonemal tubulin from very distant species, ranging from ciliated protozoa to primates. However, in contrast to polyglutamylation (Bré et al., 1994), polyglycylation was not observed in Euglenozoa, a group of ‘early emerging’ protists, suggesting that polyglycylation arose later than polyglutamylation in the course of evolution.

The high conservation of polyglycylation in motile organelles such as cilia or flagella from a very broad range of species led us to search for an involvement of this PTM in specific buffers depending upon the use.

Animals

For immunocytochemical and biochemical analyses, testes of *Drosophila melanogaster*, gonads of freshwater snail (* Biomphalaria glabrata*, Planorbidae), testes and epididymes of sexually active mice (*Mus musculus*), lemur (Prosimians, *Eulemur fulvus*; Museum National d’Histoire Naturelle, Parc Zoologique de Paris) and from human donors were also used. *Euglena gracilis* cells were employed for biochemical analyses.

**Cell isolation and protein extracts**

Mouse testes and epididymes were lacerated with forceps and spermatozoa were collected in phosphate-buffered saline (PBS) at pH 7.4. The sperm suspension was gently aspirated and diluted in PBS containing protease inhibitors (20 μg/ml leupeptin and pepstatin, 150 μg/ml benzamidine, 50 μg/ml aprotinin and ovomucoid trypsin inhibitor, 1 mM PMSF, 1 μg/ml α-phenanthroline), before being pelleted for 5 minutes at 1,000 g. One aliquot of the resuspended spermatozoa was frozen at −80°C and kept at −20°C for subsequent immunofluorescence and the rest was used for electrophoresis. Freshwater snail gonads and seminal vesicles from *Drosophila* were collected in PME buffer (PBS, pH 7.4, 2 mM MgCl$_2$, 10 mM EGTA) containing protease inhibitors. Trout spermatozoa were obtained by pressing the ventral face of the animal. Sea urchin spermatozoa were collected in a plastic Petri dish at 4°C following injection of 0.5 M KCl into the intracoelomic cavity, and were used for immunofluorescence and for the preparation of axonemes. Boar spermatozoa were used directly for immunofluorescence, or pelleted for 5 minutes at 1,000 g and resuspended in MES buffer (80 mM MES, pH 6.8, 1 mM EGTA, 0.5 mM MgCl$_2$). Either whole cells or isolated flagella from *Euglena* cells (Bré et al., 1986) were used.

**Preparation of sea urchin axonemes**

Sperm suspensions were concentrated about 2-fold by centrifugation at 750 g for 10 minutes at 4°C. The loose sperm pellet was diluted with 3 volumes of demembranation medium (DM buffer) containing 0.15 M K acetate, 0.1 mM EDTA, 1 mM DTT, 0.1% Triton X-100 and 20 mM Tris-HCl, pH 8.0. Heads and tails were detached following 12 strokes with a loose piston (type B) in a Dounce glass homogenizer. The resulting suspension was diluted 10-fold with 0.15 M K acetate, 1 mM EGTA, 1 mM DTT and 20 mM Tris-HCl, pH 8.0 (buffer A), and laid onto 15% Percoll in buffer A. After centrifugation at 2,500 g for 10 minutes, the upper layer containing the axonemes was collected, pooled and centrifuged at 10,000 g for 20 minutes. The pelleted axonemes were resuspended in DM buffer and the same procedure was repeated. Finally, the axonemes were washed twice with buffer A and the final axonemal pellets were resuspended in specific buffers depending upon the use.

**Preparation and characterization of Paramecium axonemal tubulin peptides**

*Paramecium* axonemal tubulin was extracted from cilia as described (Geuens et al., 1989), and digested with endoproteinase Asp-N. Digestion products were separated by high-performance liquid chromatography (HPLC) on a DEAE anion-exchange column and further purified by HPLC on a C8-RP300 reversed-phase column prior to amino acid sequencing and matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF-MS), as previously described (Redeker et al., 1994). For dot-blot analysis, 20 pmol of the peptides contained in each purified fraction, in ~0.1% trifluoroacetic acid (TFA), 50% acetonitrile, were concentrated by speed vacuum in the presence of sodium phosphate buffer at pH 7.4 in order to recover the peptides in 1 μl of 0.5 M phosphate buffer.

**Chemical synthesis of peptides**

Linear peptides were synthesized by the continuous flow, fluoren-9-ylmethoxycarbonyl (Fmoc)-polyamide based solid phase method (Altheron and Sheppard, 1989) on Fmoc-Pepsyn KR resin (Novabiochem). Na-Fmoc deprotection was monitored on-line during peptide assembly in an LKB ‘Biolyx’ 4170 automated synthesizer programmed to perform acylation reactions (in DMF) for 45 minutes, and Fmoc deprotection reactions (in 20% piperidine/DMF, v/v) for 10 minutes.

In addition to the standard protections of main chain termini and of threonine, aspartate and glutamate side chains, intermediates were protected from inter-main chain hydrogen bond-driven aggregation, by the synthesis of derivatives with N-(2-hydroxy-4-methoxybenzyl) (Hmb) amide backbone substitutions at strategic glycine residues (Johnson et al., 1993; Quiell et al., 1994). The synthesis of polyglycylated tubulin peptides will be reported in detail elsewhere (M. Quiell et al., unpublished). In brief, the following linear intermediates were prepared:

(1) Fmoc-β-tubulin$_{242-427}$ (AcHmbGly$_{343,440}$)-OH;
(2) Fmoc-β-tubulin$_{242-427}$ (AcHmbGly$_{343,440}$) (Glu$_{337}$-Pfp)-OH.
In general, after standard deprotections and cleavage from the resin, all Hmb-protected intermediates could be purified by preparative, reversed-phase HPLC (Vydac 208TP1022 C8, 25 cm × 2.5 cm column; aq. TFA-acetonitrile elution gradient). Key intermediates were analyzed by amino acid analysis (AAA; Beckman 7300 with ion-exchange resin, after hydrolysis in HCl), by analytical HPLC (Brownlee Aquapore RP300 C8, 250 cm × 4.6 cm column; aq. TFA-acetonitrile gradient), and by MALDI-TOF-MS in negative mode.

The unmodified, linear product H-β-tubulin\(^{27-422}\)-(OH) (β peptide) obtained from depuration of intermediate (1) gave the expected target values by AAA and MALDI-TOF-MS, within experimental errors. The assembled, fully deprotected branched target product, H-β-tubulin\(^{27-422}\)-(Glu\(^{37}\)-Gly\(^{20}\)-OH)-(OH) (β-20 Gly peptide), obtained by depuration of (4), gave a single species by MALDI-TOF-MS at m/z = 2936.8 (required [M-H] = 2938.3). AAA gave the following ratios: Asp, 1.00 (1), Thr, 0.86 (1), Glu/Gln, 9.23 (9), Gly, 21.32 (22), Ala, 2.09 (2), Phe, 0.91 (1). Sequence analysis (Edman) gave no identifiable peak at cycle 11, confirming the modification site as Glu\(^{37}\).

Lyophilised synthetic peptides were resuspended in 4 mM NaOH and kept at –20°C before use.

**Antibodies**

The monoclonal antibodies, AXO 49, TAP 952, and TAP 9311, raised against *Paramaecium* axonemal tubulin (Callen et al., 1994), have been used as hybridoma supernatants, or affinity-purified by means of Affi-Prep Protein A MAPS II kit from Bio-Rad (Ivy-sur-Seine, France). For AXO 49, ascitic fluid was also collected from Balb/c mice injected intraperitoneally with one secreting clone (prepared by Dr R. Jeannine-Wolf). Monoclonal antibodies DM1A and DM1B, directed against α- and β-tubulin, respectively (Bloet al., 1984), were purchased from Amersham (Les Ulis, France). The monoclonal antibody YL 1/2, directed against tyrosylated α-tubulin (Kilmartin et al., 1982), was purchased from Sera-Lab (Interchim, Montluçon, France). Affinity-purified rabbit anti-Glu and anti-Tyr antibodies or murine 1A2 antibody, directed against deacetylated and tyrosylated α-tubulin, respectively (Kreis, 1987), were generously provided by Dr T. E. Kreis (Geneva University, Switzerland). The monoclonal gold, TT335, directed against glumylated tubulin (Wolff et al., 1992), was a kind gift from Dr A. Wolff and Dr B. de Néchaud (Collège de France, Paris). The polyclonal anti-2-β-tubulin antibody (Paturle-Lafanechère et al., 1991) was a kind gift from Dr L. Paturle-Lafanechère (CEN, Grenoble, France). The affinity-purified rabbit antibodies, C105 and C140, directed against a carboxy-terminal and an amino-terminal sequence of β-tubulin, respectively (Arevalo et al., 1990), were kindly provided by Dr J. M. Andreu (Centro de Investigaciones Biologicas, Madrid, Spain). The monoclonal antibody 6-11B-1, directed against acetylated α-tubulin (Piperno and Fuller, 1985), was purchased from Sigma (Saint-Quentin-Falavier, France). Fluorescein-3-, Texas Red- and peroxi-dase-labeled sheep anti-mouse or goat anti-rabbit antibodies were from Diagnostics Pasteur (Marnes-la-Coquette, France).

**Fixation, inclusion and sections of *Drosophila* testes**

Gonads of males were collected in PME buffer and fixed for 2 hours at room temperature in a mixture of 3% paraformaldehyde (Prolabo, France) and 0.2% glutaraldehyde (70% EM grade, Polysciences Inc, Warrington, PA) in PME buffer. They were then briefly washed in PME, treated for 10 minutes with 1 mg/ml NaBH\(_4\) in PBS at pH 8.0, briefly washed in PME before embedding in 4% gelatin (180 bloom, Fluka AG, Switzerland) and dehydrated in graded 25% to 95% ethanol solutions. Testes were infiltrated in LR White resin (Medium grade, London Resin Company Ltd, England) in 95% ethanol (1:3, 2:2, 3:1) for 2 hours under agitation, and then overnight in a batch of resin at room temperature under agitation (Newman et al., 1983; Newman, 1987). After having been transferred in a new batch of LR White resin for 2 hours at room temperature under agitation, they were finally embedded in fully filled and tightly capped gelatin capsuls, and polymerized at 60°C for 48 hours.

Seminith (2 μm) sections of LR White embedded testes were obtained with an LKB ultrotome.

**Immunofluorescence**

Sea urchin spermatozoa were fixed as suspensions in 2% formaldehyde and permeabilized with 0.5% Triton X-100, as previously described (Gagnon et al., 1994). Drops of the sperm suspension diluted in PBS were air-dried on glass coverslips and dipped in meathanol at –20°C for 5 minutes. They were then washed in PBS plus 0.1% Triton X-100 and processed for simple and double immunolabeling. For double staining, the coverslips were successively covered with AXO 49 ascitic fluid (1:2×10\(^5\)) or TAP 952 (1:10) for 30 minutes, anti-Tyr (1:10\(^5\)) for 30 minutes, Texas Red-labeled goat anti-mouse (1:10\(^5\)) and fluorescein-labeled goat anti-rabbit (1:10\(^5\)) for 15 minutes, followed each time by PBS plus 0.1% Triton X-100. Hoechst dye 332581 (2 μg/ml, Sigma) was added in the last washing step. The antibody dilution buffer was PBS, pH 7.4, containing 3% BSA and 0.1% Triton X-100.

Spermatozoa from the other species were air-dried on glass coverslips. Mouse spermatozoa were successively fixed in meathanol at –20°C for 5 minutes, again air-dried, dipped in acetone at –20°C, washed in PME, saturated with a PME-milk solution (2.5% dried milk, Régilait, France) for 3×10 minutes and immunolabeled as above. The other spermatozoa were fixed for 5 minutes in a solution of 4% parafomaldehyde prepared in PME plus 0.5% Triton X-100, and transferred in the same solution but without Triton for 20 minutes. The cells were further washed for 2×15 minutes in PME and treated for 20 minutes with 100 mM NH\(_4\)Cl in PME. After washings in PME and saturation, they were immunolabeled as above for 45 minutes with the primary antibody and then 30 minutes with the secondary antibody. The coverslips were mounted in Aqua-Poly/mount or in Mowiol.

Sections of *Drosophila* testes were collected on polylysine-coated slides and immersed in PBS containing 50 mM NH\(_4\)Cl for 15 minutes. They were saturated in PBS containing 20 mM glycine and 3% BSA (Fraction V, protease free, Pentex Miles, France) at room temperature for 30 minutes and incubated for 2 hours with different antibodies diluted in the saturation solution: anti-Glu (1:20), anti-Δ2-tubulin (1:500), IAP 1/2 (1:50), 6-11B-1 (1:50), AXO 49 supernatant (1:2), TAP 952 (1:2) and GT335 (1:10\(^\text{2}\)). After five washes with the same buffer, the sections were incubated for 1 hour with the fluorescein- or Texas Red-labeled secondary antibody (1:10\(^\text{2}\)). The slides were then rinsed five times with PME and mounted in Citifluor (London). The last washing step contained Hoechst (1 μg/ml).

Coverslips and slides were examined with a Zeiss microscope. Micrographs were recorded on Kodak TMY 400 and Fujicolor iso 400 films. In Figs 5 and 6, two pictures of the same field were taken successively for one fluorochrome (Texas Red or fluorescein) and for Hoechst staining, without moving the film forward, in order to obtain the superimposition of the two stainings on the same picture.

**Dot-blot analysis**

*Paramaecium* tubulin and synthetic peptides were covalently bound to Immobilon-AV affinity membrane (Millipore, Saint-Quentin-en-Yvelines, France), according to the method of Canas et al. (1993), but with modifications: the membrane was first incubated for 1 hour, under agitation, in a solution (1 ml/cm\(^2\)) of 50 mg/ml 4-dimethyl aminopyrindine (Janssen Chimica, Geel, Belgium) in anhydrous acetone plus 5% tetraethylene glycol dimethyl ether (Merck, Schuchardt, Germany), in order to activate the free chemical groups of the membrane responsible.
for the covalent binding. The membrane was then quickly washed in the same solvent and stored for a short time in a dessicator before use.

For direct immunodots, 20 pmoles of each of the peptides, in 0.5 M sodium phosphate, pH 7.4, supplemented with 0.2 M NaCl, were dotted on the preactivated membrane. After air-drying and three 10 minute washings with the phosphate buffer, the membrane was saturated for 2 hours in a solution of 10% monoethanolamine (Merck) diluted in the same buffer and then treated with 0.1% Tween-20 in PBS, pH 7.4 for 45 minutes. The membrane was subsequently incubated for 2 hours with TAP 952 antibody (1:50) or AXO 49 ascitic fluid (1:10^3) diluted in PBS containing 0.3% BSA and 0.1% Tween-20. After extensive washings with the same buffer, the dots were incubated with peroxidase-labeled secondary antibody (1:2×10^3). Detection was performed by enhanced chemiluminescence (ECL, Amersham). Exposure times were from 5 seconds to 2 minutes.

For competitive immunodots, dilutions of competitor peptides were mixed with AXO 49 supernatant (1:100, containing 0.2 µg/ml antibody) or TAP 952 antibody (1:10^3) in a BSA-saturated microtiter plate and incubated overnight at 4°C. A 50 µl sample from each dilution was transferred onto a nitrocellulose membrane dotted with 0.05 to 0.005 µg Paramecium axonemal tubulin. Incubation was performed for 75 minutes at 4°C. Dots were subsequently processed with peroxidase-labeled secondary antibody and detection was carried out by ECL.

**Gel electrophoresis and immunoblotting**

Spermatozoa (from trout and mammals), gonads (from freshwater snail), seminal vesicles (from Drosophila), axonemes (from sea urchin) and Euglena cells were resuspended in hot Laemmli buffer, and boiled for 3 minutes; proteins were separated on a 10% polyacrylamide mini-gel (Laemmli, 1970), containing 0.1% (w/v) SDS (Sigma, France), at pH 9.3, and electro-transferred onto nitrocellulose, according to the method of Kyhse-Andersen (1984). The blots were stained with Ponceau red, washed in PBS, saturated with PBS containing 3% BSA and 0.1% Tween-20, and then incubated with TAP 952 (1:10^2), AXO 49 supernatant (1:10^3) or ascitic fluid (1:5×10^3 to 1:2×10^5), DM1A (1:5×10^3), DM1B (1:3×10^3), C140 (1:2.5×10^3), TAP 9311 (1:100), or GT335 (1:5×10^4) diluted in PBS containing 0.3% BSA and 0.1% Tween-20. After extensive washings with the same buffer, blots were incubated with peroxidase-labeled anti-mouse or anti-rabbit antibody and processed for ECL detection.

**Fig. 1.** Characterization of the Paramecium axonemal tubulin digestion products. (A) Glycosylation level (determined by mass spectrometry) of the peptides collected from the DEAE chromatography (number of fractions indicated in abscissa) and further purified by reversed-phase HPLC. The figure shows that the fractions were heterogeneous and contained groups of peptides differing only by a few added glycol units. (B) Reactivity of AXO 49 and TAP 952 with these peptides. The relative intensities of the immunodots are indicated. (C) Immunodot analysis of the reversed-phase HPLC purified peptides contained in fractions 2 to 10 of the DEAE chromatography. Twenty pmoles of each of the peptides were bound to the membrane and probed with AXO 49 and TAP 952. The nature of the peptides and the number of the fractions from the DEAE column are indicated on the left of each pair of dots. Two parts (denoted α1 and α2) from fraction no. 6 were enriched either in α1- or in α2-tubulin isotype (see Dupuis, 1992). Their respective reactivities with each antibody are similar whatever the α-tubulin isotype.
Demembranation and reactivation of sea urchin spermatozoa

Demembranation and reactivation of sea urchin spermatozoa were performed as previously described (Cosson and Gagnon, 1988). Under these conditions, more than 90% of the spermatozoa were motile for up to 30 minutes.

Analysis of motility parameters from demembranated-reactivated sea urchin spermatozoa

Flagellar beat frequency, wave propagation and beat amplitude were investigated using dark field microscopy with a stroboscopic light of variable frequency (Chadwick-Hellmuth, CA). Video recordings were made with a Panasonic CCD video camera WV-F15E/S-VHS, a Hamamatsu video-enhancer (DVS 3000) and a Panasonic (AG7330/S-VHS) recorder. Direct photographs of reactivated sperm axonemes were also obtained using an Olympus OM2 camera using a 1 second exposure at a flash frequency of 4 Hz.

RESULTS

Two monoclonal antibodies, AXO 49 and TAP 952, recognize polyglycylated tubulin

In order to determine the chemical nature of the epitopes recognized by AXO 49 and TAP 952, two complementary approaches were chosen: probing the antibodies with peptides derived from their natural antigen, i.e. Paramecium axonemal tubulin, and probing them with synthetic peptides.

First, in the aim of studying the reactivity of our antibodies with natural tubulin peptides, Paramecium axonemal tubulin extracted from cilia was proteolyzed, the carboxy-terminal digestion products were purified, and analyzed by amino acid sequencing and mass spectrometry (Fig. 1A), as previously described (see Materials and Methods, and Redeker et al., 1994). In parallel, the peptides from the purified fractions were covalently bound to a membrane and immunoprobed with AXO 49 and TAP 952 (Fig. 1C); the results of these analyses are summarized in Fig. 1A,B. TAP 952 recognized all the polyglycylated peptides with different affinities. In contrast, AXO 49 strongly recognized the highly glycylated α- and β-tubulin peptides and not those bearing lower levels of glycyla-
tion (up to about 5 glycyl units for α- and 8 glycyl units for β-tubulin peptides). The specific detection of differently glycylated tubulin peptides with either AXO 49 or TAP 952 (Fig. 1B) indicates that these two antibodies recognize polyglycylated tubulin peptides differing in the level of glycyla-
tion.

Second, in order to ascertain the specificity of the two antibodies for polyglycylated tubulin, we synthesized two peptides mimicking the carboxy-terminal end of β-tubulin. One consists of the 16 amino acid residues of the carboxy-terminal end of β-tubulin (β: 427DATAAEEGFEFEEEQG445), the other has the same sequence but with 20 glycyl residues on Glu 437 (β-20 Gly). We underline that the synthesis of this class of branched peptides represents a chemical challenge. The difficulty comes from the tendency of homopolymers to form poorly solvated β structured inter and intramolecular aggregates during synthesis, either in solution or on solid phase. We have therefore used a recently developed backbone amide protection strategy for the temporary solubilisation of intermediates during peptide synthesis (Johnson et al., 1993) and have applied it here to the synthesis of a branched polyglycylated β-tubulin peptide.

The β and β-20 Gly synthetic peptides were immunoprobed with AXO 49 and TAP 952. As expected, neither antibody rec-
ognized the bare terminal sequence (Fig. 2A, β), even when used at very low dilutions. In contrast, both antibodies interacted with β-20 Gly; however, the reactivity of TAP 952 was lower than that of AXO 49 (Fig. 2A, β-20 Gly).

Competitive experiments between Paramecium axonemal tubulin dotted on a membrane and each synthetic peptide showed that, in contrast to the β peptide, β-20 Gly was an effective competitor for the binding to AXO 49 and TAP 952 (Fig. 2B). The interaction of Paramecium tubulin with both antibodies was partially abolished by 50 μM of polyglycylated peptide and completely by 100 μM. However, with TAP 952, a lower amount of tubulin than in the case of AXO 49 was needed to observe the inhibition of binding to tubulin.

Two factors can account for the different behaviour of the complexes between the glycylated peptide and each antibody. (1) In axonemal tubulin, a greater number of glycylated isoforms is reactive with TAP 952 than with AXO 49 (see Fig. 1B). Therefore, the same amount of tubulin is expected to be more efficient to dissociate the complex with TAP 952 than with AXO 49. (2) The dissociation constant of the complex of β-20 Gly with TAP 952 would be higher than with AXO 49, which fits well with the idea of a better interaction of AXO 49 than of TAP 952 with the highly glycylated peptides. This lower stability of the TAP 952-peptide complex could also be accounted for by a greater sensitivity of TAP 952 to the epitope conformation, the latter being probably altered in the synthetic peptide. Notice that other authors have shown that the reactivity of a synthetic tubulin peptide, α(415-443), with the DM1A

Fig. 2. Immunoreactivities of AXO 49 and TAP 952 antibodies with tubulin synthetic peptides. (A) Dot-blot analysis of AXO 49 and TAP 952 reactivities with each of the synthetic peptides: β-20 Gly and β. The number of pmol of each of the peptides dotted on the membrane is indicated on the left. (B) Competitive immunodot of AXO 49 and TAP 952 with the two synthetic peptides: β-20 Gly and β. Axonemal tubulin amount per dot: 0.05 μg for AXO 49 and 0.015 μg for TAP 952. Given the lack of inhibition of reactivity of both antibodies by the β peptide, the reactivity of only one of them (AXO 49) is shown in the presence of this peptide.
anti-sequence antibody was comparable to that of tubulin, whereas the reactivity of another synthetic peptide, β(412-431), with the DM1B antibody was much lower than that of tubulin, these authors suggesting therefore a contribution of the conformation of β-tubulin in the interaction with the antibody, not contained in the β-tubulin synthetic peptide (de la Viña et al., 1988). Is is worth mentioning that the concentrations of the latter peptide and of the glycylated β-tubulin peptide efficient for competition in each system are similar.

In summary, the immunoreactivity results provided both from Paramecium tubulin peptides and from synthetic peptides demonstrate that AXO 49 and TAP 952 are directed against polyglycylated tubulin, and indicate that the two antibodies recognize isoforms differing in at least the level of glycylation.

Polyglycylated tubulin is widespread among the axonemes from phylogenetically distant species

Polyglycylated tubulin was initially found in axonemal tubulin from Paramecium (Redeker et al., 1994). The two antibodies, AXO 49 and TAP 952, recognizing different levels of glycation, represent powerful tools to examine the presence of polyglycylated tubulin in axonemes from different species.

For this purpose, we chose flagellated protozoa and spermatozoa from various metazoan species and probed axonemal tubulin by immunoblotting with AXO 49 and TAP 952 (Fig. 3). For each species, α- and β-tubulins were, respectively, detected with DM1A and DM1B, two universal antibodies directed against conserved sequence epitopes, and in some cases, glutamylated tubulin with GT335 antibody. As representatives of protists, we used various lineages, in which the Euglenozoa are known from molecular phylogenies (Sogin, 1991) to be among the earliest ones to emerge. The metazoa included a mollusc (freshwater snail), an echinoderm (sea urchin), and several vertebrates. Within vertebrates, trout represented the fishes, and ram, boar, mouse and primates, the mammals. Within the primates, lemur and man were chosen as respective representatives of early and late emerging lineages.

In the flagellated protozoan Euglena gracilis (Fig. 3) and in Trypanosoma (not shown), tubulin was labeled with GT335 but was neither stained with AXO 49 nor with TAP 952. In most other species, both tubulin subunits were specifically recognized by AXO 49 and TAP 952. In freshwater snail and ram, AXO 49 and TAP 952 displayed a higher reactivity with β-tubulin than with α-tubulin. In sea urchin and trout, AXO 49 preferentially reacted with α-tubulin, while the recognition of the β subunit was better with TAP 952. Spermatozoa from the majority of mammals tested were labeled with both antibodies (Figs 3 and 5a-c), except in man where α- and β-tubulins were stained with TAP 952 but not with AXO 49 (Figs 3 and 5d-f). Compared to the lemur, in human, the lack of reactivity of AXO 49 (Fig. 3 and Fig. 5b,e) suggests that a decrease in polyglycylation level has occurred between an early emerging primate and man.

In conclusion, our results show the distribution of polyglycylated tubulin on a very broad evolutionary range, the affinities of AXO 49 and TAP 952 for α- and β-tubulin depending upon the species.

Because sea urchin spermatozoa possess an easily accessible axoneme, with none of the accessory structures found in mammalian spermatozoa, they were chosen for double immunostaining with AXO 49 or TAP 952, together with a polyclonal anti-Tyr antibody. While the latter uniformly labeled the length of the axoneme (Fig. 6a), AXO 49 showed a gradient of reactivity along the whole length, starting from the sperm head and decreasing towards the axonemal tip (Fig. 6b). Interestingly, an opposite gradient was observed with TAP 952; the reactivity decreased from the tip towards the head (Fig. 6c). These observations are indicative of an uneven distribution of the polyglycylated tubulin isoforms along the axoneme of sea urchin spermatozoa.

Polyglycylation is the last tubulin posttranslational modification detected in the course of spermatozoan maturation

Sperm cells of drosophilids offer a favourable system to study
the kinetics of appearance of tubulin polyglycylation compared to the other PTMs, since sperm maturation occurs with high synchrony within individual cysts and the stage of maturation can be identified cytologically. In *Drosophila melanogaster*, groups of 64 spermatids develop in parallel within the same cyst, prior to the appearance of the flagellar membrane which individualizes spermatozoa at the final stage of maturation.

In a protein extract of seminal vesicles from *Drosophila* separated by SDS-PAGE, the set of polypeptide bands reactive with TAP 952 was located at the level and just above the α subunit detected by DM1A, while the bands reactive with AXO 49 were upward shifted with respect to both major tubulin bands stained by DM1A and DM1B (Fig. 7). This smear of reactive isoforms was largely detected by two other antibodies, C140 (not shown) and TAP 9311, directed against N-terminal sequences of β- and α-tubulin, respectively. It is noteworthy that the reactivity of AXO 49 is restricted to the slowest migrating isoforms stained by TAP 952.

Given the numerous PTMs already detected in spermatozoa (Hermo et al., 1991; Fouquet et al., 1994), we wondered whether some of them would be specific for certain stages of maturation. We therefore compared the distribution of ∆2-tubulin, and of acetylated, detyrosylated, glutamylated, and polyglycylated tubulins in axonemal microtubules during sper-
matogenesis in *Drosophila* with the antibodies specific for these PTMs, namely anti-Δ2 tubulin, 6-11B-1, anti-Glu antibody, GT335, AXO 49 and TAP 952, as well as with the 1A2 anti-Tyr antibody as reference. Semi-thin sections of *Drosophila* testis displaying many cysts at different stages of maturation were processed for immunofluorescence with these antibodies. As expected, all axonemal structures in the whole set of cysts were brightly stained with 1A2 (Fig. 8a). Axonemes within young and mature cysts were likewise labeled with 6-11B-1 (Fig. 8b), anti-Glu antibody (Fig. 8c), GT335 (Fig. 8e) and anti-Δ2-tubulin antibody (not shown). Comparison between serial sections labeled with anti-Glu (Fig. 8c) or with AXO 49 (Fig. 8d) antibody showed that the latter reacted exclusively with a cluster of cysts with relatively small diameter located in the central region of the two sections. The axonemes within these narrow cysts, well ordered and often hexagonally packed, correspond to cysts in late stages of spermatogenesis (Lindsley and Tokuyasu, 1980). The reactivity of TAP 952 was likewise restrictive, since it stained only the narrow cysts and not the immature ones (Fig. 8f). These results show that, among the various PTMs tested during the process of flagellar maturation in *Drosophila* spermatozoa, tubulin polyglycylation is the only PTM which takes place at the end of maturation.

**AXO 49 and TAP 952 inhibit the motility of demembranated sea urchin sperm axoneme**

As an attempt to approach the function of polyglycylation, we tested the effects of our antibodies on axonemal motility using permeabilized cell models. Demembranated sea urchin spermatozoa, reactivated by ATP in the presence of AXO 49 around 1 μg/ml, showed a progressive decrease in the percentage of motile spermatozoa as well as in their swimming rate (not shown), and changes of various swimming parameters (Fig. 9), over a 30 minute reactivation period. High concentrations of AXO 49 completely blocked axonemal motility within shorter time periods. In contrast, while a very strong labeling of axonemes was observed in sperm preparations incubated with other anti-tubulin antibodies such as DM1A, DM1B, directed to primary

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**Fig. 7.** Tubulin isoforms of seminal vesicle proteins from *Drosophila melanogaster* detected with AXO 49, TAP 952, DM1B followed by DM1A or TAP 9311, after SDS-PAGE and immunoblotting. The protein amounts correspond to 1.5 vesicles for blotting with DM1A, DM1B and TAP 9311, and 4.6 vesicles for blotting with AXO 49 and TAP 952.

**Fig. 8.** Immunofluorescence staining on sections of *Drosophila* testes. Sections were stained with anti-Tyr-tubulin antibody 1A2 (a), anti-acetylated tubulin antibody 6-11B-1 (b), anti-Glu-tubulin antibody (c), anti-polyglycylated tubulin antibodies, AXO 49 (d) and TAP 952 (f), and anti-glutamyalted tubulin antibody GT335 (e); (c and d) serial sections. Arrows, mature narrow cysts. Arrowheads, young large cysts. The 64 axonemes are conspicuous here in transverse sections of cysts at intermediate stages of maturation. Bar, 10 μm.
sequences in the carboxy-terminal part of α- and β-tubulin respectively, and YL 1/2, directed to the carboxy-terminal end of tyrosinated tubulin, no inhibition of motility was observed even at antibody concentrations above 200 μg/ml (not shown).

Analysis of individual video frames of the inhibition of sea urchin sperm motility revealed that AXO 49 progressively decreased the swimming efficiency by the combination of several effects. The major early effect was the induction of an erratic type of swimming, the head being widely displaced on both sides of the sperm trajectory; this effect, already visible at 0.5 μg/ml AXO 49, increased with antibody concentration (Fig. 9A). In addition, the proximal and distal ends of the flagellum showed a reduced beating amplitude and a lower beat frequency and became progressively paralyzed with time and with increasing AXO 49 concentration (Fig. 9B). Similar effects, consisting of the displacement of the head on both sides of the sperm trajectory followed by a decrease of beating amplitude and beat frequency, were observed with TAP 952 (not shown).

Preincubation of AXO 49 with the β-20 Gly synthetic peptide prevented the inhibition of spermatozoan motility by AXO 49 in a concentration-dependent manner; β-20 Gly was an effective competitor of AXO 49-induced head displacement (Fig. 9C).

DISCUSSION

AXO 49 and TAP 952 recognize different forms of polyglycylated tubulin

The results reported here demonstrate that our two monoclonal antibodies, AXO 49 and TAP 952, which react exclusively with tubulin in whole Paramecium cellular extracts, recognize polyglycylated α- and β-tubulin. The evidence includes the reactivity over axonemal tubulin peptides analyzed in parallel by mass spectrometry, the reactivity with a polyglycylated tubulin synthetic peptide, together with the lack of reactivity over a β chain synthetic peptide.

The different reactivities of TAP 952 and AXO 49 with α- or β-tubulin of spermatozoa from some species, their different affinities for the proximal and distal parts of sea urchin axonemes, and the lack of reactivity of only AXO 49 with human spermatozoa argue in favour of a difference in the epitopes recognized by the two antibodies. This assumption is confirmed by the immunodot analysis of the Paramecium axonemal tubulin peptides. TAP 952 recognized all the polyglycylated α- and β-tubulin peptides with different affinities. In contrast, the highest reactivity of AXO 49 was observed with the highly glycylated forms of tubulin (containing 11 to 34 and 15 to 32 glycyl units, added to the α and β subunits, respectively; Fig. 1), those bearing low levels of glycylation being not recognized. This is the likely reason why axonemal tubulin from some species such as freshwater snail (Fig. 3) or Paramecium (Levilliers et al., 1995), separated by SDS-PAGE and immunoblotted with AXO 49, displayed a smear indicating the presence of a variety of reactive isoforms with slightly higher apparent molecular masses than those of the bulk of tubulin. Conversely, in sea urchin, the lack of shift is consistent with the lower level of glycylation found in this species (J. Mary et al., unpublished).

In order to achieve the covalent bonding of the glycine residues, we presume that at least two distinct enzymes are required: one for the linkage of a glycine to a glutamate residue by a γCOOH-αNH₂ amide bond, and one for the αCOOH-αNH₂ amide linkage between the added glycine residues.
The different levels of tubulin polyglycylation are related to microtubule stability

In *Paramecium*, 13 distinct microtubular arrays have been described (Cohen et al., 1982; Cohen and Beisson, 1988) and, strikingly, the distribution of the epitopes recognized by TAP 952 and AXO 49 within the cell completely differs. AXO 49 exclusively decorates the most stable microtubules, such as those associated with the oral apparatus, the contractile vacuole system and the cilia, whereas TAP 952 decorates, in addition, an intracytoplasmic network of cold- and drug-sensitive (hence dynamic) microtubules (Fleury et al., 1995). This leads us to infer that, in *Paramecium*, cytoplasmic tubulin is polyglycylated in addition to the axonemal one, and that it differs from the latter at least in its level of glycylation. In order to check these possibilities, the analysis of *Paramecium* cytoplasmic tubulin by mass spectrometry is in progress. Moreover, the fact that dynamic and stable microtubules are differently recognized by TAP 952 and AXO 49, which are specific for different levels of glycylation, shows that a correlation between the degree of glycylation and microtubule stability exists, at least in this cell type.

Since polyglycylation reduces the apparent acidity of the carboxy-terminal domain of tubulin (Redeker et al., 1994), the different degrees of polyglycylation could serve as modulators for microtubule stability by decreasing the net charge and hydrophilicity of this region (Serrano et al., 1984; Sackett et al., 1985; Mejillano and Himes, 1991; Mejillano et al., 1992).

Given the tendency of polyglycyl homopolymers to form β structured intermolecular aggregates, it is reasonable to postulate that polyglycyl chains branched on tubulin could favour intermicrotubule interactions. This assumption is supported by the pattern of AXO 49 immunoreactivity in *Paramecium*, which is restricted to axonemes and bundles of microtubules (Fleury et al., 1995). The homopolymer (Gly)ₙ laterally branched on the tubulin subunits might also favour the interactions with microtubule-associated proteins. As a matter of fact, different kinds of interactions of glycine rich motifs in proteins have already been shown with various ligands, such as nucleotides (Krauhs et al., 1981) and RNA (Ghisolfi et al., 1992). Alternatively, the long glycyl chains in tubulin could anchor axonemal microtubules to the membrane, leading to their stabilization. Indeed, the immunoreactivity of AXO 49 has always been observed on stable microtubules that are in the proximity of membranous structures (Fleury et al., 1995).

**Tubulin polyglycylation could represent one of the last morphogenetic signals during spermatozoan maturation**

We have shown, on the one hand, that during spermatogenesis in *Drosophila*, polyglycylated tubulin, probed with both AXO 49 and TAP 952, is detected at the end of flagellar maturation. This situation is up to now unique, given that, in another model system, *Paramecium*, the delayed appearance of immunoreactivity following microtubule assembly is observed with AXO 49 but not with TAP 952 (Fleury et al., 1995), meaning that only the high levels of tubulin glycylation occur there as late events.

On the other hand, polyglycylation, among a series of PTMs, is the last one occurring in the course of sperm differentiation. Therefore we can assume that polyglycylation is developmentally regulated during spermatogenesis; this late event could constitute a signalling device, indicating to the morphogenetic ‘clock’ that the structural maturation process has been completed.

It is worth noticing that the AXO 49 epitope is always detected on microtubule networks located close to membranes (Fleury et al., 1995). Moreover, we have shown that, in sea urchin spermatozoa, the epitope is unevenly distributed along the axoneme, suggesting a polarized distribution of polyglycylated tubulin isoforms, with higher levels of glycylation in the proximal part of the axoneme. This polarized distribution is consistent with the polar growth of the flagellum. It is also reminiscent of that observed with an antibody against an outer dynein arm component (Gagnon et al., 1994). These data, taken as a whole, suggest that some of the enzymes responsible for microtubule polyglycylation could be closely associated with the ciliary or flagellar membrane and distributed according to a gradient.

**Axonemal tubulin polyglycylation arose early and is widely distributed in the course of eukaryotic evolution**

Polyglycylation represents an abundant posttranslational modification of axonemal tubulin in *Paramecium*, since all the peptides analyzed after tubulin purification were found to be polyglycylated (Redeker et al., 1994). In contrast, brain tubulin and cytoplasmic tubulin from mammalian cells are not reactive with AXO 49 and TAP 952 (Callen et al., 1994). Therefore, in order to determine the evolutionary distribution of this modification, the presence of polyglycylated tubulin was investigated with both antibodies in the axonemes of phylogenetically distant species.

From the ciliate phylum, including *Tetrahymena*, *Stylonychia* (not shown) and *Paramecium*, to the primates, axonemal tubulin was found to be polyglycylated. Whereas in most species, both tubulin subunits are reactive with both antibodies, their respective degrees of reactivity depend upon the species and are not similar for the two antibodies, indicating different ratios of the various polyglycylated tubulin isoforms in each subunit. For instance, in contrast to the 32 to 34 glycy units found in β- and α-tubulin from *Paramecium*, respectively (Redeker et al., 1994), a maximum of 13 glycy units has been recently reported in bull spermatozoa, in β-tubulin only (Rüdiger et al., 1995). From this subunit specificity, the authors emphasized a distinction between the tubulins from mammals and from the protozoan *Paramecium*. However, in the mammals we have tested, both subunits of axonemal tubulins were found to be reactive with our antibodies. Thus, the difference in glycylation levels of both subunits does not reside in a distinction between protistan and mammalian tubulins. It could be explained by a difference in the affinity of the subunits of various species for the glycylating enzymes or by the presence of two distinct sets of enzymes for the glycylation of α- and β-tubulins.

In human spermatozoa, the lack of reactivity of AXO 49 only indicates an absence of the highly glycylated isoforms found in the other species. This could result from the absence or the inhibition of some of the enzymes, given that the glutamate residues potentially involved in the lateral branching of the polyglycyl chains are present in tubulin from man, as from other species (Little and Seehaus, 1988). Alternatively,
an equilibrium between two enzymatic activities of glycylation and deglycylation could regulate the length of the polyglycyl chain. In Euglena cells, although tubulin was found to be glutamylated, the lack of reactivity of both AXO 49 and TAP 952 suggests an absence of polyglycylated tubulin, assuming that AXO 49 and TAP 952 are able to detect the whole family of polyglycylated isoforms.

In summary, a wide variation in reactivity towards the two anti-polyglycylated tubulin antibodies is observed both across species and between the α and β subunits. Thus, in order to validly draw inferences about the presence of polyglycylation, the use of both antibodies in combination is crucial.

We conclude that polyglycylation arose early in eukaryotic evolution but later than glutamylation (see Bré et al., 1994), and that the enzymatic machinery responsible for this modification has been conserved over a very broad evolutionary scale, but is lacking or different in primitive eukaryotes and is modified in human spermatozoa.

Since polyglycylation is specific to cilia and flagella over a wide range of species and is the last modification occurring among a series of other PTMs during spermato genesis, we postulate that it could either represent a morphogenetic signal for the end of ciliary and flagellar maturation or that it could be involved in the process of motility. However, some lineages, such as the Euglenozoa, do not exhibit any reactivity towards AXO 49 and TAP 952. Thus, if polyglycylation is decisive during the differentiation process or in motility of cilia or flagella, one has to assume that at least two different systems would have arisen to perform this role in the course of evolution.

Involvement of polyglycylated tubulin in spermatozoan motility

AXO 49 and TAP 952, by interacting with specific tubulin posttranslational epitopes, interfere with flagellar motility of sea urchin spermatozoa, inducing a specific pattern of inhibition of motility which consists of a head path widening in addition to a reduction of beat amplitude and frequency. These effects are observed at antibody concentrations in the μg/ml range. Unrelated antibodies as well as anti-tubulin sequence antibodies directed against different epitopes located just a few amino acids apart from the glycylation sites and used at concentrations two orders of magnitude higher than AXO 49 or TAP 952 have no effect on sperm motility. Moreover, the effects of some anti-tubulin antibodies on sea urchin sperm motility, consisting of a decrease of flagellar bend angles only but not of beat frequency, have been interpreted as indirect ones on axonemal microtubules (Asai and Brokaw, 1980; Asai et al., 1982). Indeed, the ATP-dependent dynein-tubulin mechanochemical cycle is primarily responsible for ciliary and flagellar beating (Gibbons and Gibbons, 1972; Gibbons, 1981, 1989; Yano and Miki-Noumura, 1981; Johnson, 1985), and any modification of dynein activity leads to an alteration of beat frequency. As observed for anti-dynein antibodies (Gagnon et al., 1994), AXO 49 reduces the frequency of beating; thus it may interfere with the interaction between dynein and tubulin on the microtubular doublet. In this respect, the length of the polyglycyl chain could be used as a spatial tag detected by dynein for a precise recognition of its specific binding site on the microtubule subfiber B during the mechanochemical cycle involved in movement.

Compared with the inhibition patterns of other anti-tubulin antibodies on flagellar movement (Gagnon et al., unpublished), AXO 49 and TAP 952 display a unique effect, namely the widening of the head path, leading to an erratic type of swimming. A more detailed analysis of the wave pattern at high magnification and using high frequency video framing at 300-800 Hz is in progress. It indicates that both antibodies induce several perturbations in the axoneme that are reminiscent of those induced by Ca2+ ions, i.e., an asymmetry of beating combined with a progressive dampening of the wave propagation along the axoneme (Brokaw et al., 1974). As the sea urchin sperm flagellum contains several types of Ca2+ sensors (Brokaw, 1991), interference of AXO 49 or TAP 952 with motility could also occur at the site of interaction between tubulin and one of these calcium sensors.

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