The A and B Tubules of the Outer Doublets of Sea Urchin Sperm Axonemes Are Composed of Different Tubulin Variants†

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ABSTRACT: The α*β*-tubulin heterodimer, the structural unit of microtubules, comes in many variants. There are different α and *β* isotypes encoded by multigene families. Additional heterogeneity is generated by a set of posttranslational modifications. Detyrosination of α-tubulin, removal of the carboxy-terminal Glu-Tyr dipeptide of α-tubulin, phosphorylation of some tubulins, polyglutamylation, and polyglycylation of α- and *β*-tubulins all involve the acidic carboxy-terminal region. We have investigated the distribution of tubulin variants in the axonemal microtubules of sea urchin sperm flagella by immunological procedures and by direct sequence and mass spectrometric analysis of the carboxy-terminal peptides. The A and B tubules that comprise the nine outer doublets differ strongly in tubulin variants. A tubules contain over 95% unmodified, tyrosinated α*β*-tubulin. In B tubules, α-tubulin is ∼65% detyrosinated and both α- and *β*-tubulin are 40−45% polyglycylated. These results show a segregation of tubulin variants between two different axonemal structures and raise the possibility that posttranslational modifications of tubulins reflect or specify structurally and functionally distinct microtubules.

Microtubules are involved in many different cellular functions of eukaryotes. Their major structural unit, the α*β*-tubulin heterodimer, displays many variants. Diversity of α*β*-tubulin is generated genetically through the presence of distinct α and *β* genes and additionally through a set of posttranslational modifications. Except for the acetylation of lysine at position 40 in some α-tubulins (L'Hernault & Rosenbaum, 1983, 1985), all currently known modifications are located in the highly acidic carboxy-terminal peptides of α- and *β*-tubulin [reviewed in Mary et al. (1994) and Rüdiger et al. (1995)]. The cyclic detyrosination and tyrosination of α-tubulin depends on a presumptive tubulin carboxypeptidase and the well-characterized tubulin tyrosine ligase [reviewed in Greer and Rosenbaum (1989) and Ersfeld et al. (1993)]. Removal of tyrosine and the penultimate glutamic acid residue yields Δ2 α-tubulin, which is no longer a substrate for the ligase (Paturle-Lafanechère et al., 1991; Rüdiger et al., 1994). Both α- and *β*-tubulins are also subject to polyglutamylation. The lateral chain, which can reach some eight residues, is connected via an isopeptide bond formed by the *γ*-carboxylate of a particular glutamic acid residue. The position of this modified residue has been identified in all brain tubulins (Eddé et al., 1990; Alexander et al., 1991; Paturle-Lafanechère et al., 1991; Redeker et al., 1992; Rüdiger et al., 1992; Mary et al., 1994). Finally, polyglycylation has been demonstrated for α- and *β*-tubulin in the ciliary axonemal microtubules of the protist *Paramecium tetraurelia* (Redeker et al., 1994) and for the *β*-tubulin of bull sperm flagella (Rüdiger et al., 1995).

The functional role of tubulin modifications is still poorly understood. In dynamic cytoplasmic microtubules, modifications seem related to microtubular turnover. Modified tubulins (detyrosinated α-tubulin, Δ2 α-tubulin, and acetylated α-tubulin) accumulate in polymers with slow turnover (Schulze et al., 1987; Wehland & Weber, 1987a; Paturle-Lafanechère et al., 1994). However, the long lived axonemal microtubules of embryonic sea urchin cilia still contain large amounts of tyrosinated α-tubulin in addition to detyrosinated α-tubulin (Stephens, 1992). In this case, a diversity in tubulin variants can hardly reflect microtubular turnover and may instead be related to some aspects of a specialized microtubular structure and/or function.

Axonemes, the complex microtubular assemblies of cilia and flagella, are among the most ancient and widespread tubulin structures. Although the molecular mechanisms driving their assembly are not fully understood, their conserved 9+2 structure offers three well-defined classes of microtubules. Nine outer doublet microtubules are arranged around the central pair of single microtubules. Each outer doublet contains a complete 13-protofilament A tubule and an incomplete 11-protofilament B tubule fused together so that A and B tubules share 5 protofilaments (Alberts et al., 1994). In sea urchin sperm flagella, tubulin molecules from each class of axonemal microtubules can be isolated by

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established fractionation procedures (Norrander & Linck, 1993).

Here, we have examined the distribution of different carboxy-terminal posttranslational modifications of tubulin in axonemal microtubules from sea urchin sperm axonemes. We have obtained intact A tubules separated from remnants of B tubules. Immunological procedures and direct chemical characterization of the carboxy-terminal tubulin peptides revealed striking differences between A and B tubules. While B tubules are extensively detyrosinated and polyglycylated, the A tubules contain unmodified tubulin. This segregation of tubulin variants in twin microtubule assemblies suggests a role of posttranslational modifications in the morphogenesis of axonemal doublets.

MATERIALS AND METHODS

Purification and Fractionation of Sea Urchin Sperm Axonemes. Demembranated sperm flagellar axonemes from the sea urchin *Paracentrotus lividus* were prepared and fractionated as described by Multigner et al. (1992). The successive treatments with 0.6 M KCl, the 5 min exposure to 40 °C, and the sequential extractions using 0.5% sarkosyl and 0.5% sarkosyl/2 M urea in TED buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 1 mM DTT] followed precisely the previous protocol. At each step, soluble and insoluble fractions were separated by centrifugation (100000*g*, 1 h). The remaining insoluble material was devoid of tubulin (Multigner et al., 1992; Norrander & Linck, 1993).

Isolation of Sarkosyl Resistant Tubulin Assemblies. Axonemes were KCl extracted and heat treated as described above. The remaining insoluble material was resuspended in 20 volumes of TED buffer containing 0.5% sarkosyl, left on ice for 30 min, and loaded on a discontinuous sucrose gradient in TED buffer. In a typical experiment, a 250 μL aliquot of sarkosyl-treated material was loaded at a final protein concentration of 2 mg/mL on a gradient composed of successive layers of TED buffer containing 20% sucrose (490 μL), 50% sucrose (490 μL), and 70% sucrose (100 μL). Centrifugation was at 4 °C in a Beckman TLA rotor (45 000 rpm, 45 min). The pellet was resuspended in 200 μL of TED buffer and either processed for microscopy or stored at −80 °C for further use. In control experiments, the KCl and heat resistant axonemal fractions in TED buffer were directly loaded on a sucrose gradient without prior exposure to 0.5% sarkosyl and processed as described above. The KCl and heat resistant structures are designated as A^+ tubules, while the sarkosyl resistant structures derived from A^+ tubules are designated as A tubules (for explanation of these designations, see Results).

Purification of α- and β-Tubulins for Peptide Analysis. Proteins from different axonemal fractions (see Results) were recovered by trichloroacetic acid precipitation. Pellets were washed twice with cold acetone. In the first set of experiments, precipitated proteins were solubilized in 6 M guanidine hydrochloride prior to reverse phase HPLC and 200 μg were loaded onto a Baker-Bond C_8 wide pore analytical column (300 Å, 4.6 mm × 250 mm) equilibrated in 10% acetonitrile containing 0.1% TFA.[1] Elution, monitored at 215 nm, was with a linear gradient of acetonitrile (10 to 70%) in 0.1% TFA at a flow rate of 1 mL/min. All relevant fractions were dried under N_2, solubilized in sample buffer, and subjected to 8% SDS−PAGE. Following staining with Coomassie brilliant blue, bands corresponding to α- or β-tubulin were cut out. Gel slices were destained, extensively washed with distilled water, and kept at −20 °C until use. Approximately 50−100 μg of purified α- and β-tubulin were obtained per axonemal fraction. In the second set of experiments, precipitated proteins from axonemal fractions were directly dissolved in sample buffer and subjected to SDS−PAGE. α- and β-tubulin bands were processed as described above.

Isolation of Carboxy-Terminal Tubulin Peptides. Gel bands containing α- or β-tubulin (see above) were processed to allow the isolation of the entire carboxy-terminal region carrying the various posttranslational modifications (Rüdiger et al., 1995). For α-tubulin, this is readily achieved by cleavage with endoproteinase LysC at the last lysine residue, which lies in a highly conserved sequence. As β-tubulins lack a similarly placed lysine and would yield much larger fragments of some 55 residues, we preferred fragmentation with CNBr, since the last methionine residue lies in a highly conserved sequence.

α-Tubulin present in 10−15 stained gel pieces was concentrated into a small band using the agarose-based gel concentration system of Rider et al. (1995). Treatment with endoproteinase LysC (Boehringer Mannheim, Germany) at 3 μg/mL was in 0.1 M Tris-HCl (pH 8.5), 5% in acetonitrile, for 16 h at 37 °C. Stained gel pieces containing β-tubulin were processed for CNBr cleavage as described (Rüdiger et al., 1995).

Using a SMART (Pharmacia, Uppsala, Sweden) fast-desalting column, tubulin digests were recovered in buffer A (20 mM sodium phosphate, pH 7.0) and separated on a MonoQ column (1.6 × 50 mm) equilibrated in buffer A with a 2.4 mL linear salt gradient (0 to 0.5 M NaCl in buffer A) followed by a 0.9 M salt wash. The flow rate was 100 μL/min. Elution profiles were monitored by absorption at 214 nm, and 100 μL fractions were collected. Some peak fractions were analyzed by automated sequencing and mass spectrometry (see below). Except for the trailing edge of the last peak, which contains the polyglutamylated species, the peak fractions were combined and subjected to reverse phase HPLC on a narrow-bore Vydac 218 TP52 column (2.1 × 250 mm). 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 was 0.1% TFA. Solvent B was 70% acetonitrile in 0.08% TFA. Elution was monitored at 214 nm using an Applied Biosystems 759A absorbance detector. Peptides were collected manually and characterized (see below). Fractions from the trailing edge of the MonoQ column were also combined and subjected to reverse phase HPLC.

Characterization of the Carboxy-Terminal Tubulin Peptides. Peptides were sequenced by automated Edman degradation using an Applied Biosystems gas phase sequenator (model 470) or Knauer sequenators (models 810 and 910). All instruments were equipped with on-line phenylthiohydantoin amino acid analysis. In the highly glycylated peptides from the B tubule, some positions established as glutamic acid in the unmodified peptides provided no phenylthiohydantoin amino acid. Since the sequence con-

[1] Abbreviations: mAb, monoclonal antibody; MALDI, matrix-assisted laser desorption ionization; TFA, trifluoroacetic acid.

tinued normally past these positions, they reflect a glutamic acid with an oligo glycyl side chain.

Mass spectra were recorded with a KRATOS MALDI 3 time of flight mass spectrometer (Shimadzu, Duisburg, Germany) using α-cyano-4-hydroxycinnamic acid in 30% acetonitrile and 0.06% TFA as a matrix. Spectra of negative ions were recorded in the linear mode. Calibration was with bovine insulin and α-melanocyte-stimulating hormone. In some cases, peptides were also characterized by amino acid composition.

Antibodies and Immunofluorescence Microscopy. Rabbit polyclonal antibodies directed either against detyrosinated α-tubulin (Glu α-tubulin) or detyrosinated α-tubulin lacking the penultimate glutamic acid (Δ2 α-tubulin) were a generous gift from Dr. L. Paturle-Lafanechère. In addition, three monoclonal antibodies to α-tubulin were used. Murine mAb TU.01 (Monosan, Uden, The Netherlands) recognizes the amino-terminal domain. Murine mAb 6-11B-1 (Sigma Chemical Co., St. Louis, MO) is specific for acetylated α-tubulin. The rat mAb YL$^1/_2$, a generous gift from Dr. J. V. Kilmartin, reacts only with tyrosinated α-tubulin (Tyr α-tubulin). Axonemal tubulin assemblies were centrifuged on glass coverslips, fixed in methanol at −20 °C for 6 min, and processed for immunofluorescence. In some experiments, the methanol fixation step was omitted as indicated in the figure legends. Double staining for total α-tubulin and Glu α-tubulin was with mAb TU.01 and polyclonal anti-Glu tubulin as primary antibodies and rhodamine-conjugated goat F(ab′)$_2$ fragment anti-mouse IgG (Jackson, ImmunoResearch Laboratory, West Grove, PA) and FITC-conjugated goat F(ab′)$_2$ fragment anti-rabbit IgG (Jackson, ImmunoResearch Laboratory) as secondary antibodies. Double staining for total α-tubulin and Tyr α-tubulin was with mAbs TU.01 and YL$^1/_2$ as primary antibodies and rhodamine-conjugated goat F(ab′)$_2$ fragment anti-mouse IgG and FITC-conjugated goat F(′ab′)$_2$ fragment anti-rat IgG as secondary antibodies (Jackson, ImmunoResearch Laboratory).

Detyrosination and Tyrosination of Axonemal Tubulin Assemblies. In detyrosination experiments, whole axonemes (2.9 mg/mL) or purified A tubules (0.86 mg/mL) were treated with pancreatic carboxypeptidase A (Serva, Heidelberg, Germany) at a final concentration of 10 μg/mL for 10 min at 30 °C. Detyrosination was stopped by the addition of DTT to a final concentration of 20 mM (Paturle et al., 1989). Tubulin tyrosine ligase was purified as described (Ersfeld et al., 1993). Brain tubulin, serving as a control, and outer doublets isolated from carboxypeptidase-treated axonemes were used for tyrosination experiments (Paturle et al., 1989).

Other Methods. Proteins were separated by SDS−PAGE using 8% gels and stained with Coomassie brilliant blue or transferred to nitrocellulose sheets. Blots were exposed to various α-tubulin antibodies, followed by alkaline phosphatase-conjugated secondary antibodies (Promega Corp., Madison, WI) and phosphatase substrates. Electron microscopy was as in Saoudi et al. (1995).

RESULTS

Distribution of Tyr, Glu, and Δ2 α-Tubulin in Axoneme Fractions. We have previously designed methods yielding homogeneous preparations of Glu, Tyr, and Δ2 α-tubulins which can be used as standards for the semiquantitative estimation of these α-tubulin derivatives in crude protein extracts (Paturle-Lafanechère et al., 1994). Using these procedures, we found that axonemes are composed of tyrosinated and detyrosinated α-tubulin in nearly equal amounts and that they also contain a small proportion of Δ2 α-tubulin (not shown). We next tested whether these tubulins are evenly distributed among axonemal microtubules or whether subcellular sorting of tubulin variants occurs in the different types of axonemal microtubules. We also analyzed the distribution of acetylated α-tubulin.

Axonemal proteins were fractionated using sequential treatments with 0.6 M KCl, high temperature (40 °C), and detergent (0.5% sarkosyl and 0.5% sarkosyl in 2 M urea) as described in Materials and Methods, and the corresponding protein profiles are shown Figure 1A. Lane 1 contains the KCl-soluble fraction which includes the tubulin from the central pair microtubules (Gibbons & Fronk, 1971; Farrell & Wilson, 1978). Immunoblot analysis (Figure 1B) shows that all three carboxy-terminal α-tubulin variants as well as acetylated α-tubulin are present in the KCl-soluble fraction. KCl treatment of axonemal structures provides intact outer doublet microtubules which are isolated by centrifugation (Gibbons & Fronk, 1971; Farrell & Wilson, 1978). Lane 2 of Figure 1A shows the protein profile of the heat sensitive components of outer doublets which include most of the tubulin from B tubules (Stephens, 1970). Corresponding immunoblots (Figure 1B) show that B tubules contain all the tubulin variants. Heat treatment of the outer doublets leaves A tubules associated with some B tubule remnants (Linck, 1976; Linck & Langevin, 1981). These structures were isolated by centrifugation. They were progressively solubilized by successive treatments with 0.5% sarkosyl (lanes 3 and 4) and 0.5% sarkosyl in 2 M urea (lane 5). Examination of the corresponding protein profiles shows that the first sarkosyl treatment solubilizes a protein fraction similar in composition to the fraction corresponding to B tubules. It again contains all α-tubulin variants (Figure 1B, lane 3). A further treatment with sarkosyl releases a protein fraction which still contains B tubule proteins but shows in addition an enrichment for two major components of about 77 and 83 kDa (lane 4). The remaining resistant structures (lane 5) have been described as sheets of protofilaments from the A tubules (Meza et al., 1972; Witman et al., 1972; Linck, 1976; Norrander & Linck, 1993). They contain in addition to tubulin the tektins and the 77 and 83 kDa polypeptides. Both tubulin as well as the 77 and 83 kDa polypeptides can be solubilized by the addition of 2 M urea to the sarkosyl solution (Linck & Langevin, 1982; Linck & Stephens, 1987). Immunoblot analysis shows an unexpected distribution of tubulin variants. The tubulin present in fraction 5 reacts with acetylated α-tubulin antibodies but lacks reactivity with Glu and Δ2 α-tubulin antibodies. It reacts more strongly than any other axonemal fraction with mAb YL$^1/_2$ which is specific for tyrosinated α-tubulin (Figure 1B, lane 5). These results show that sarkosyl treatment of the heat resistant structures derived from outer doublets selects for a tubulin assembly uniquely enriched in tyrosinated α-tubulin. Glu and Δ2 α-tubulins are not found in this fraction but are present in all other axonemal fractions tested.

Sarkosyl Treatment of Heat Resistant Outer Doublets Yields Integral A Tubules. Previous work and this study (see below) showed that heat treatment of sperm flagellar outer doublets results in the isolation of A fibers associated with B tubule remnants (Linck, 1976). In the following text, we

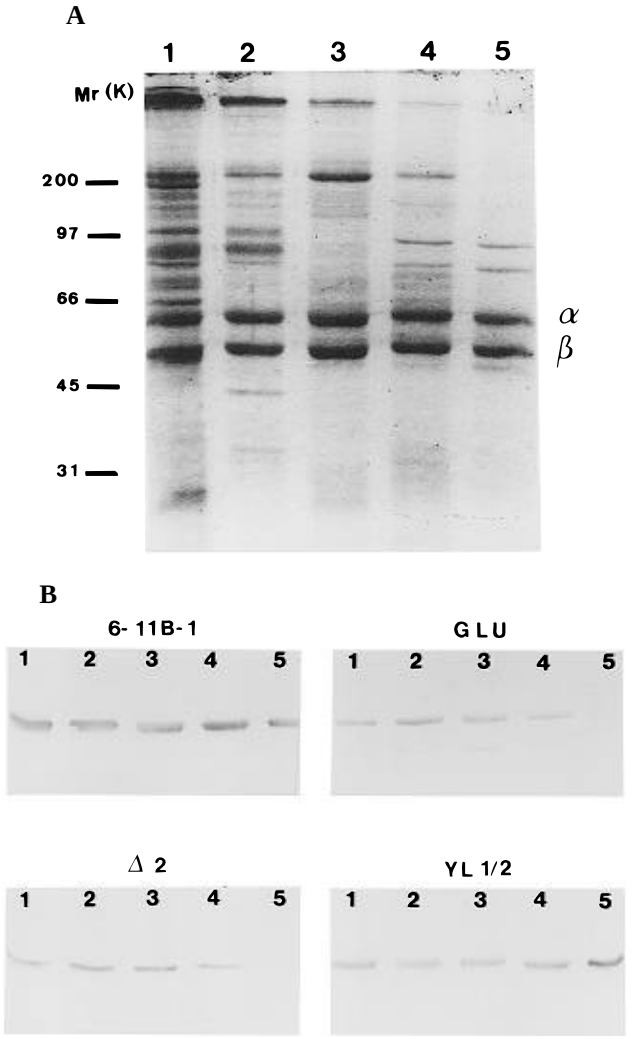

FIGURE 1: Analysis of α-tubulin variants in different axonemal fractions. (A) Coomassie brilliant blue-stained 8% gel. The numbers at the left indicate M_r value in thousands. Lanes 1−5 show the polypeptide composition of the following axonemal fractions: lane 1, 0.6 M KCl-soluble fraction; lane 2, heat-soluble fraction; lanes 3 and 4, sarkosyl-soluble fractions (two successive extractions with 0.5% sarkosyl); and lane 5, 0.5% sarkosyl in the 2 M urea-soluble fraction. Samples of each fraction contained about 2 μg of tubulin as estimated from gel scans using pure tubulin as a standard (not shown). The positions of α- and β-tubulin are marked. (B) Immunoblot analysis for the lanes shown in panel A. Proteins transferred to nitrocellulose sheets were treated with α-tubulin antibodies mAb 6-11B-1, anti-Glu α-tubulin, anti-Δ2 α-tubulin, and mAb YL$^1/_2$ as indicated. Secondary antibodies were conjugated to alkaline phosphatase for enzymatic visualization. mAbs 6-11B-1 and YL$^1/_2$ react with acetylated and tyrosinated α-tubulin, respectively. Note the presence of acetylated and tyrosinated α-tubulin in all fractions, while detyrosinated and Δ2 α-tubulins are not detected in fraction 5, which reacts particularly strongly for tyrosinated α-tubulin.

shall refer to these structures as A^+ tubules. Previous work also showed that 0.5% sarkosyl treatment of axonemal structures yields sheets of protofilaments (Meza et al., 1972; Witman et al., 1972; Linck, 1976; Norrander & Linck, 1993). Since the sequence of treatments described above was a modification of existing protocols, we examined the structures generated by 0.5% sarkosyl treatment of A^+ tubules in more detail. A^+ tubules were isolated by heat treatment of outer doublets as described in Materials and Methods. Pelleted A^+ tubules were resuspended in TED buffer and either saved for further biochemical and microscopical analysis or treated with 0.5% sarkosyl. Sarkosyl resistant assemblies were centrifuged onto a discontinuous sucrose gradient in TED buffer. The sucrose gradient step was introduced to stabilize tubulin structures during centrifugation. The pelleted material was resuspended in TED buffer and analyzed in parallel with A^+ tubules.

Panels a and d of Figure 2 show the protein composition of A^+ tubules and the sarkosyl resistant structures derived from them. Panels b, c, and e−g of Figure 2 show the corresponding electron microscopical images. In agreement with previous work (for references, see above), A^+ tubules showed a high proportion of tubules of the A type associated with B tubule remnants (Figure 2b,c). Interestingly, the sarkosyl resistant structures derived from A^+ tubules showed in negative staining a homogeneous population of filamentous structures. Cross sections provided images of apparently normal microtubules with 13 protofilaments. Some intraluminal electron dense moieties were observed. We conclude that sarkosyl treatment of A^+ tubules mainly yields apparently intact tubules of the A type (Figure 2e−g). Therefore, we will refer to these structures as A tubules.

Immunofluorescence Analysis of A^+ and A Tubules. Immunofluorescence microscopy was used to monitor the yield of A tubules purified from the A^+ tubule fraction and to assess directly the presence or absence of α-tubulin variants. Pellets of A^+ tubules were resuspended in TED buffer and split into two equal aliquots. One aliquot was directly sedimented in a sucrose gradient. The second aliquot was treated with sarkosyl prior to sedimentation in a sucrose gradient. Pellets containing A^+ and A tubules were resuspended in the same volume of TED buffer, and equal aliquots were sedimented onto glass coverslips and processed for immunofluorescence. Staining of A^+ and A tubules with mAb TU.01 yielded similar patterns of fibers, either isolated or somewhat aggregated (Figure 3). No obvious difference was apparent with regard to the total amount of material on the coverslip or to the length of A^+ and A fibers, suggesting that most A^+ tubules were converted to A tubules following exposure to sarkosyl. When A^+ and A tubules were stained with Glu α-tubulin antibody, a striking difference was seen. The antibody brightly stained A^+ tubules but gave no signal on A tubules. This result strongly suggests that A fibers do not contain Glu α-tubulin and that the Glu α-tubulin signal in the A^+ tubules arose from the B tubule remnants attached to the A fibers. Additional experiments using the YL$^1/_2$ antibody, specific for tyrosinated α-tubulin, showed as expected bright staining of both A^+ and A fibers (data not shown).

The combined immunoblot and immunofluorescence analysis suggests a major difference in α-tubulin variants for A and B tubules. While B tubules show clear detyrosination, A tubules seem mainly composed of tyrosinated tubulin. Chemical analysis of the carboxy-terminal peptides of α- and β-tubulin was used to obtain more detailed information on the state of modifications of the tubulin molecules in A and B tubules.

A and B Tubules Differ in Posttranslational Modifications of α- and β-Tubulins. α- and β-tubulins from the different axonemal fractions were subjected to chemical analysis following purification as described in Materials and Methods. Tubulin bands from stained SDS−PAGE were used to obtain the carboxy-terminal peptides. Cleavage with endoproteinase LysC was used in the case of α-tubulin, while β-tubulin was

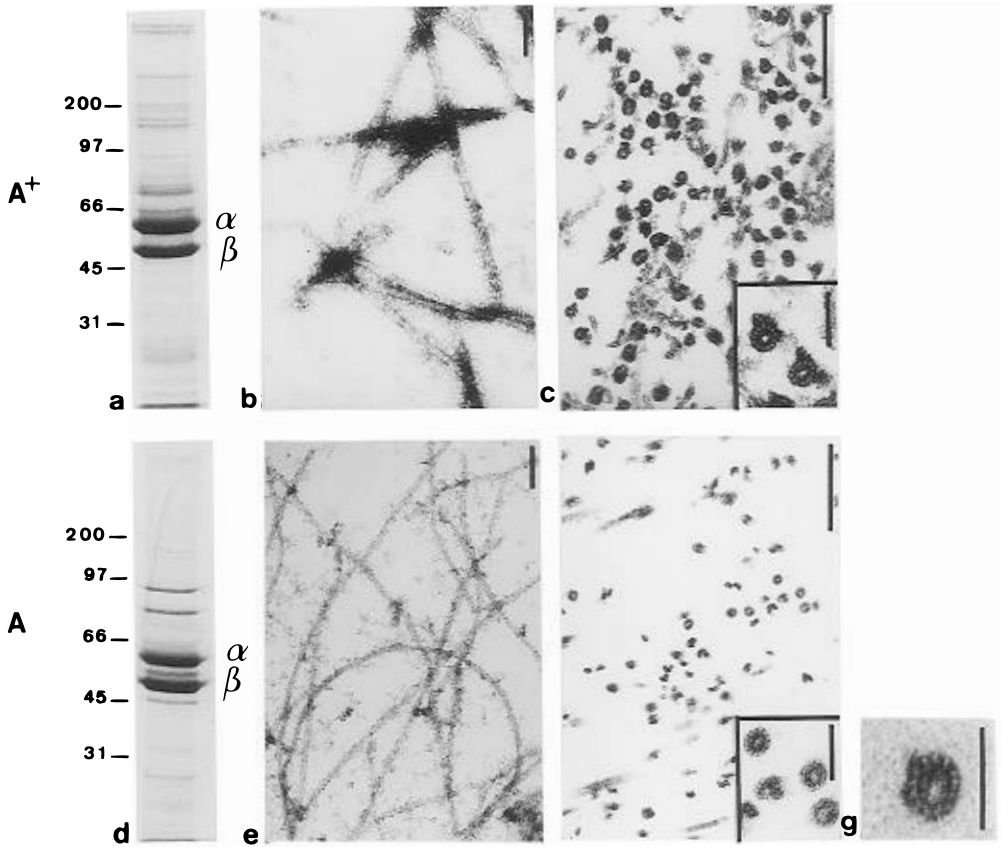

FIGURE 2: Protein composition and ultrastructure of the heat resistant A^+ tubules and of the sarkosyl resistant A tubules from axonemes. A^+ (a−c) and A (d−g) tubules were isolated on sucrose gradients (Materials and Methods). (a and d) Coomassie brilliant blue-stained gels of A^+ (a) and A (d) tubules. The positions of α- and β-tubulins are indicated. (b and e) Electron micrographs of negatively stained A^+ (b) and A (e) tubules. (c, f, and insets) Electron micrographs of thin sections of A^+ (c) and A (f) tubules at two different magnifications. (g) High-magnification image of a sectioned A tubule. The bars in panels b, c, e, and f are 200 nm long. The bars in the insets and g are 50 nm long.

subjected to CNBr cleavage. Anion exchange chromatography on a small MonoQ column separated the very acidic carboxy-terminal peptides from all other fragments. Except for the trailing edge containing the polyglutamylated species, all other species were pooled and subjected to HPLC separation. Figure 4A provides as an example the HPLC profile of the carboxy-terminal α-tubulin peptides from the purified A tubules.

Aliquots from the MonoQ and HPLC profiles were first screened by mass spectrometry. Fractions lacking modifying groups were used for automated sequencing. In agreement with its mass of 2362.8, the unmodified carboxy-terminal α-tubulin peptide of A tubules (Figure 4B) has the sequence DYEEVGVDSVEGEAEEEGEEY (Figure 5). Although this sequence of *Pa. lividus* differs from various sea urchin α-tubulins in the data base, it is in perfect agreement with a partial cDNA sequence for a testis specific α-tubulin sequence reported by Alexandraki and Ruderman (1983) for the sea urchin *Lytechnicus pictus* (Genbank number PO2553). The unmodified β-tubulin peptide from A tubules has a molecular mass of 3669 which fits the sequence following the last methionine in the β-tubulin cDNA clone isolated by Di Bernardo et al. (1989) from an embryonic cDNA library of *Pa. lividus* (Genbank number X15389): NDLVSEYQQYQDATAEEEGEFDEEEEGDEEAA. Automated sequencing of the unmodified peptide of β-tubulin provided exactly this sequence except that of the two final alanine residues only the first residue was reached (Figure 5). Several minor and one moderately abundant β-peptide from the B tubule showed a carboxy-terminal trimming involving one to four residues (Table 1) versus the proposed master sequence. Due to incomplete cleavage at the last methionine, we also isolated additional fragments which start after the penultimate methionine of the β-tubulin sequence of Di Bernardo et al. (1989).

All fractions from the HPLC elution profiles were analyzed by mass spectrometry, and Table 1 summarizes the combined results. Both α- and β-tubulin peptides from A tubules were very homogeneous populations. About 95% of the carboxy-terminal peptides reflected the unmodified proteins. In the case of α-tubulin, this is the tyrosinated form (Figure 4). Trace amounts involved the detyrosinated form and the species lacking both tyrosine and the penultimate glutamic acid residue. A trace amount of a species carrying a single glutamic acid residue as a side chain was also detected (for a summary, see Table 1 and Figure 4). In the case of β-tubulin, the unmodified form accounted again for more than 95%. Trace amounts involved a species trimmed by the two carboxy-terminal alanines, which is also observed as the sole polyglutamylated form (Table 1).

The B tubule material yielded an entirely different result for both α- and β-tubulin (Table 1). About 40−45% of both tubulins were polyglycylated with the number of side chain glycine residues ranging from one to eleven in α-tubulin and one to eight in β-tubulin. Polyglycylation involved in α-tubulin only the detyrosinated form, while in β-tubulin, this modification was found on the normal β-tubulin and its derivative lacking the three carboxy-terminal residues. Thus,

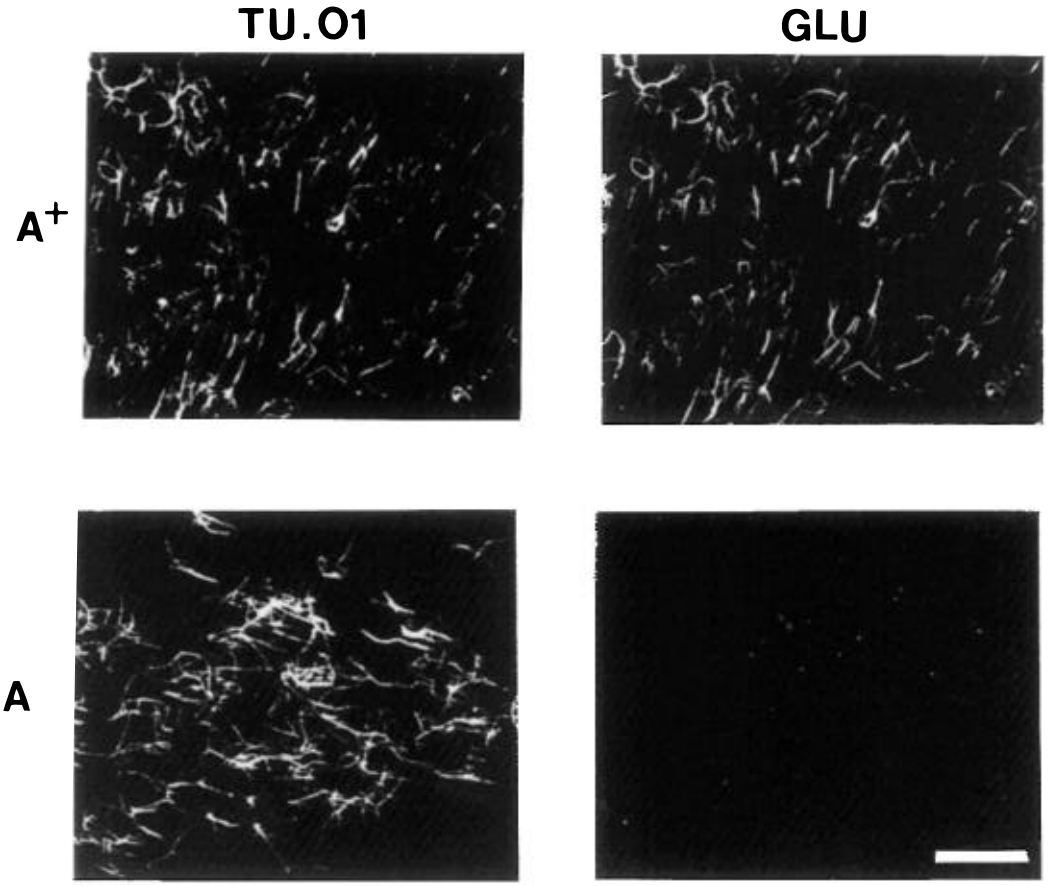

FIGURE 3: Immunofluorescence analysis of Glu α-tubulin abundancy in A^+ and A tubules. A^+ and A tubules were double stained for α-tubulin with mAb TU.01 and anti-Glu tubulin as indicated. Since mAb TU.01 reacts with a conserved region in the amino-terminal domain of α-tubulin, this antibody was used in positive controls. The anti-Glu tubulin is specific for detyrosinated α-tubulin. Glu tubulin is abundant in A^+ tubules and undetectable in A tubules. Bar = 10 μm.

there are three major α-tubulin species in the B tubule: tyrosinated α (~35%), detyrosinated α (~25%), and detyrosinated and polyglycylated α (~40%). In the corresponding β-tubulin, the three major species are unmodified β (~50%) and polyglycylated β with a normal (~25%) and a slightly shortened carboxyl end (~20%). Additional species occurring in trace amounts, which include the glutamylated forms, are listed in Table 1.

Tubulin in the salt-soluble axonemal fraction also showed posttranslational modifications (not shown). Since salt extraction was performed on relatively crude material and not on isolated central pair microtubules, additional studies on these structures will be necessary to establish the degree of tubulin modification. Analysis of the sarkosyl-soluble fractions derived from A^+ tubules showed a peptide composition similar to that of heat-soluble tubulin from B tubules (data not shown), suggesting that B tubule remnants attached to A tubules are similar in composition to the bulk of the B tubule material.

In an attempt to locate the position(s) of the polyglycine side chain(s), automated Edman degradation was repeated on highly modified carboxy-terminal peptides from the B tubule (Figure 5). Since the discovery of polyglutamylation in mammalian brain tubulin (Eddé et al., 1990), the position of the first residue in the carboxy-terminal peptides which is undetected by Edman degradation has been taken as the residue carrying the polyglutamate side chain (for references, see the introductory section). Using the same argument, Redeker et al. (1994) proposed that in ciliary axonemes of *Paramecium* the polyglycyl chains are attached to glutamic acids 437 and 445 in β- and α-tubulin, respectively. They emphasized, however, that additional polyglycylation sites in the carboxy-terminal peptides could not be excluded. Our sequences on the highly glycylated peptides from the B tubule of sea urchin sperm axonemes favor two (α) and three (β) neighboring glutamic acid residues as the modified residues (Figure 5). They occupy positions in the carboxy-terminal sequences related to the single sites identified so far in *Paramecium* tubulin. These residues lie in a stretch of acidic residues flanked by A or G and G (α-tubulin) and F and G (β-tubulin), respectively. The alignment also shows that an aspartic acid residue following the phenylalanine in sea urchin β-tubulin is not used by the glycylation enzyme, which seems to recognize specifically particular glutamic acid residues (Figure 5).

The Carboxy-Terminal End of α*-Tubulin Is Accessible in Isolated Axonemes and A Tubules.* A possible explanation for the lack of detyrosinated α-tubulin in A tubules could be an A tubule specific inaccessibility due to some associated protein which is absent in B tubules. Two experiments showed that at least in isolated axonemes and native A tubules the carboxy-terminal end of α-tubulin is accessible. First, demembranated axonemes were treated with pancreatic carboxypeptidase A under standard conditions for detyrosination. Subsequent extraction steps as in Figure 1 yielded fractions morphologically and biochemically identical to those observed in normal axonemal fractions. A tubules were isolated from normal and carboxypeptidase-treated

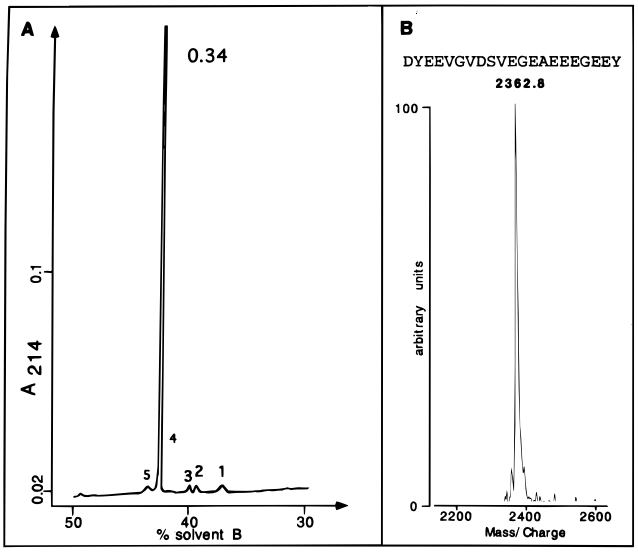

FIGURE 4: Characterization of the carboxy-terminal peptides of α-tubulin from the purified A tubules. α-Tubulin purified by SDS−PAGE was digested with endoproteinase LysC. The acidic carboxy-terminal peptides isolated by MonoQ chromatography were pooled and analyzed by reversed phase HPLC (A). All fractions were characterized by mass spectrometry. No peptide material was present in peak 1. Peak 2 contained trace amounts of the carboxy-terminal peptide from tyrosinated and detyrosinated α-tubulin (ΔY) and α-tubulin lacking both tyrosine and the penultimate glutamic acid (ΔYΔE). Peak 3 contains trace amounts of the tyrosinated peptide carrying one extra glutamic residue as a side chain. Peak 4, which is off scale, is the tyrosine containing unmodified peptide. Trace amounts of this peptide also elute in peak 5. Panel B shows the MALDI mass spectrum obtained on peak 4 of panel A. Note the single mass/charge of 2362.8 which matches exactly the sequence (top in panel B) of the unmodified α-tubulin peptide determined by automated Edman degradation of the same sample. Thus, at least 95% of the α-tubulin from A tubules lacks posttranslational modifications in the carboxy-terminal region (Table 1).

axonemes, and the proteins from both preparations were analyzed on immunoblots. Figure 6A shows that the α-tubulin was fully detyrosinated due to the earlier exposure of the axonemes to carboxypeptidase A. When detyrosinated A tubules were exposed to tubulin tyrosine ligase, no detectable incorporation of tyrosine was found (data not shown). Second, we tested whether normal A tubules bind mAb YL$^1/_2$ without previous methanol fixation. Purified A tubules were incubated in parallel with mAb YL$^1/_2$ and mAb TU.01 with or without an initial methanol fixation step. Methanol-fixed A tubules reacted with both mAbs (Figure 6B). Native A tubules, not previously fixed with methanol, did not react with mAb TU.01, which shows no reaction with native tubulin (Grimm et al., 1987). On the other hand, mAb YL$^1/_2$ reacted strongly with A tubules whether or not a methanol fixation step was used (Figure 6B).

DISCUSSION

The A and B tubules of axonemal outer doublet microtubules are twin tubulin assemblies differing in geometry and some binding proteins [reviewed in Alberts et al. (1994)]. The different thermal stability of A and B tubules in sea urchin sperm outer doublets (Stephens, 1970) allows for a simple fractionation of the tubulins in the two structures. Using antibodies specific for certain α-tubulin derivatives in immunofluorescence microscopy and immunoblotting experiments, we observed a strong difference in tubulin

α tubulin	
S.U. sperm	DYEEVGVDSVEGEAEEEGEEY
Param.	DYEEVGIETAEGEGE-EGEA
β tubulin	
S.U. sperm	NDLVSEYQQYQDATAEEEGEFDEEEEGDEEAA
Param.	NDLVSEYQQYQDATAEEEGEFEE--EGEQ
B. sperm	NDLVSEYQQYQDATAEEEGEFEE--EAEEEVA

FIGURE 5: Sequence alignments of the carboxy-terminal regions of α- and β-tubulins, for which polyglycylation has been established. The sequences are for axonemes of sperm flagella from the sea urchin (S.U.), *Pa. lividus* (this study), the ciliary axonemes of the protist *P. tetraurelia* (Param.) (Redeker et al., 1994), and β-tubulin from bull sperm (B. sperm) (Rüdiger et al., 1995). Only one of the two highly similar α-tubulins of *Paramecium* is shown. The sea urchin sequences were obtained on the unmodified carboxy-terminal peptides of α- and β-tubulin from the A tubule. The last alanine residue of β-tubulin predicted by mass spectrometry is in line with cDNA sequence information (Di Bernardo et al., 1989), although it was not reached by automated Edman degradation. In the highly polyglycylated peptides of α- and β-tubulins from the B tubule, no phenylthiohydantoin derivative was obtained at positions involving an underlined E. These positions are thought to carry an oligoglycine side chain via an isopeptide bond formed by the γ-carboxylate of the particular glutamic acid residue. The corresponding information on *Paramecium* tubulins is also given. Although Redeker et al. (1994) documented only one modified residue, they specifically state that additional sites of polyglycylation are not excluded. Note the relative alignment of the modified glutamic acid residues. In the case of the polyglycylated bull sperm β-tubulin, the position of the modified residue(s) was not determined (Rüdiger et al., 1995).

variants (Figures 1 and 3). Although both tubules contain acetylated and tyrosinated α-tubulin, all the detyrosinated α-tubulin is restricted to the B tubule. Direct sequence and mass spectrometric characterization of the carboxy-terminal peptides quantitated these data and revealed an additional striking difference in tubulin variants. Tubulin polyglycylation is restricted to the B tubules, where it involves both α- and β-tubulin to 40−45%. Thus, A tubules are built from αβ-tubulin essentially devoid of carboxy-terminal modifications, while B tubules contain extensively detyrosinated (α) and polyglycylated (α and β) tubulins (Table 1).

The segregation of certain tubulin variants poses some interesting questions regarding the mechanism of outer doublet morphogenesis. Provided that polymer formation involves only tyrosinated tubulin, the subsequent action of the α-tubulin carboxypeptidase must somehow be restricted to B tubules, which are 60% detyrosinated, and leave the A tubules with their full complement of tyrosinated tubulin unchanged. Alternatively, if both tyrosinated and detyrosinated tubulin participate in assembly, there must be a mechanism which prevents the detyrosinated form from entering the A polymer permanently, since at least the normal tyrosine tubulin ligase seems to act only on the αβ heterodimer and not on microtubules (Thompson, 1982; Wehland & Weber, 1987b). Currently, we do not know how the segregation of tubulin variants takes place. Although the carboxy-terminal end of α-tubulin in isolated axonemes and A tubules is readily available to pancreatic carboxypeptidase and mAb YL$^1/_2$ (Figure 6), the *in vivo* situation could be different due to a binding protein protecting the tubulin ends in the A tubule. The second segregation of tubulin

Table 1: A and B Tubules of Sea Urchin Sperm Flagellar Axonemes Differ in Posttranslational Modifications of α- and β-Tubulin[a]

α-tubulin			β-tubulin	
B	A		B	A
Y	34%	>95%	unmodified	50%	>95%
ΔY	24%	ta	unmodified + poly-Gly	25%; 1−8	−
ΔY + poly-Gly	40%; 1−11	−	Δ2AΔ1E + poly-Gly	20%; 2−8	−
Y + poly-Gly	−	−	Δ1A	ta	−
Y + poly-Glu	ta; 1−6	ta; 1	Δ2A	ta	ta
ΔY + poly-Glu	ta; 1−3	−	Δ2AΔ1E	ta	−
ΔYΔE	ta	ta	Δ2AΔ2E	ta	−
			Δ1A + poly-Glu	ta; 1−3	−
			Δ2A + poly-Glu	ta; 1	ta; 1−4

[a] Summary of the posttranslational modifications identified in the isolated carboxy-terminal peptides of α- and β-tubulin. Approximate content in percent of different posttranslationally modified α- and β-tubulin derivatives in A and B tubules of sea urchin sperm flagellar axonemes. A stands for the purified A tubules (see text). Numbers following the percent value or the abbreviation ta give the number of glycine (Gly) or glutamic acid (Glu) residues in the polyglycine and poly(glutamic acid) side chain(s) (for details, see the text) detected by mass spectrometry. ta indicates the presence of trace amounts; − indicates not found. Additional changes in α-tubulin concern the presence of the carboxy-terminal tyrosine (Y), its absence (ΔY), and the absence of tyrosine plus the penultimate glutamic acid (ΔYΔE). β-Tubulin shows in addition to an unmodified main chain (unmodified) a carboxy-terminal trimming involving either one (Δ1A), two (Δ2A), three (Δ2AΔ1E), or four (Δ2AΔ2E) residues. Results on α-tubulins are from two different preparations; results on β-tubulin are from a single preparation. Note that purified A tubules contain essentially unmodified α- and β-tubulin, while the B tubules show a high degree of posttranslational modifications. Note particularly that polyglycylation is restricted to B tubules (for details, see the text). In some peptides, the number of glycine residues in the side chain reaches 13 in α and 9 in β.

variants concerns again the B tubule. Polyglycylated tubulin occurs exclusively in the B tubules, where it accounts for some 40−45% of both α- and β-tubulin (Table 1). Recent immunological results on *Drosophila* spermatogenesis indicate that polyglycylation is a late event, which takes place well after the axonemal structure is formed (Bressac et al., 1995). In contrast, detyrosination in sea urchin cilia formation seems to be an early step in axoneme formation (Stephens, 1992). Thus, it is interesting to note that polyglycylation of α-tubulin in the B tubule of sea urchin sperm axonemes seems to involve only the detyrosinated form (Table 1). This correlation between detyrosination and polyglycylation could possibly mean that, at least in sea urchin sperm B tubules, detyrosination of αβ-tubulin is a prerequisite for polyglycylation of either α- or β- or both tubulin subunits.

The chemical analysis of the carboxy-terminal peptides yielded additional interesting results. First, the major α-tubulin isotype of sperm flagella of *Pa. lividus* differed from the α-tubulins described for this sea urchin by cDNA cloning (Gianguzza et al., 1989, 1990). These cover several very similar α-tubulins and a distinct testis specific one. This leaves the question of whether the presumptive testis specific α-tubulin defined by cDNA cloning reflects a minor sperm α-tubulin. Thus, mammalian sperm shows in addition to a major α- and β-tubulin isotype several minor tubulins, some of which may even be specifically compartmentalized, such as for instance in the manchette (Hecht et al., 1988; Fouquet et al., 1994). Second, the level of polyglutamylated tubulins in the sea urchin sperm axonemal preparations monitored by mass spectrometry was low (Table 1), considering the two-dimensional immunoblots of mouse spermatazoa with mAb GT335, thought to be specific for polyglutamylated tubulin (Fouquet et al., 1994). Possible reasons for this difference include the use of different metazoan phyla, a comparison of purified flagellar axonemes with intact flagella, which seem to contain also some membrane-bound tubulin (Stephens, 1992), an uncontrolled loss of some tubulin species, and the formal possibility that sperm tubulins carry an additional polyglutamic acid substitution outside the carboxy-terminal region. Third, we found indications for an unexpected and novel processing of tubulin, again in the B tubule. Surprisingly, 20% of the β-tubulin lacked the three carboxy-terminal residues of the unmodified β-tubulin. Such a proteolytic trimming is unlikely to be the result of artifactual digestion during preparation, since it was not found in the purified A tubules despite the longer isolation procedure.

Some molecular aspects of polyglycylation, the most recently discovered posttranslational modification of tubulin (Redeker et al., 1994; Rüdiger et al., 1995), require additional study. In the ciliary axonemes of the protist *Paramecium*, polyglycylation seems to be a complete modification process, since all α- and β-tubulin molecules carry between 1 and 34 glycyl units (Redeker et al., 1994). In a partial analysis involving only some of the tubulin present in bull sperm axonemes, a much lower level of glycyl units (up to 13 residues) was found in about 60% of the β-tubulin (Rüdiger et al., 1995), and recent experiments show that the α-tubulin is also only partially glycylated (U. Plessmann and K. Weber, unpublished observation). Our detailed analysis of the A and B tubules of sea urchin sperm flagella reported here shows that polyglycylation is restricted to the B tubule, where it involves 40−45% of both α- and β-tubulin, with the number of glycyl units ranging from 1 to 11 (Table 1). Thus, the extent of the modification and the length of the side chain(s) may differ in different eukaryotes or axonemal organizations. We also note that in the sea urchin sperm tubulins from B-tubules there are two to three well-defined glycylation sites (Figure 5), while in *Paramacium* tubulins, there is a major site, although additional sites are not excluded (Redeker et al., 1994). Thus, there is clearly a need for a better understanding of the properties of the glycylation enzymes and their action on axonemes from different organisms.

The A and B tubules of axonemal outer doublets differ not only in geometry but also in some binding proteins (Alberts et al., 1994; Figure 16−41). Thus, the A tubule binds the tektins (Norrander & Link, 1993; see also Figure 1), and the radial spokes are exclusively attached to the A tubules (Curry & Rosenbaum, 1993). Other differences concern the action of ciliary dynein, the major motor protein

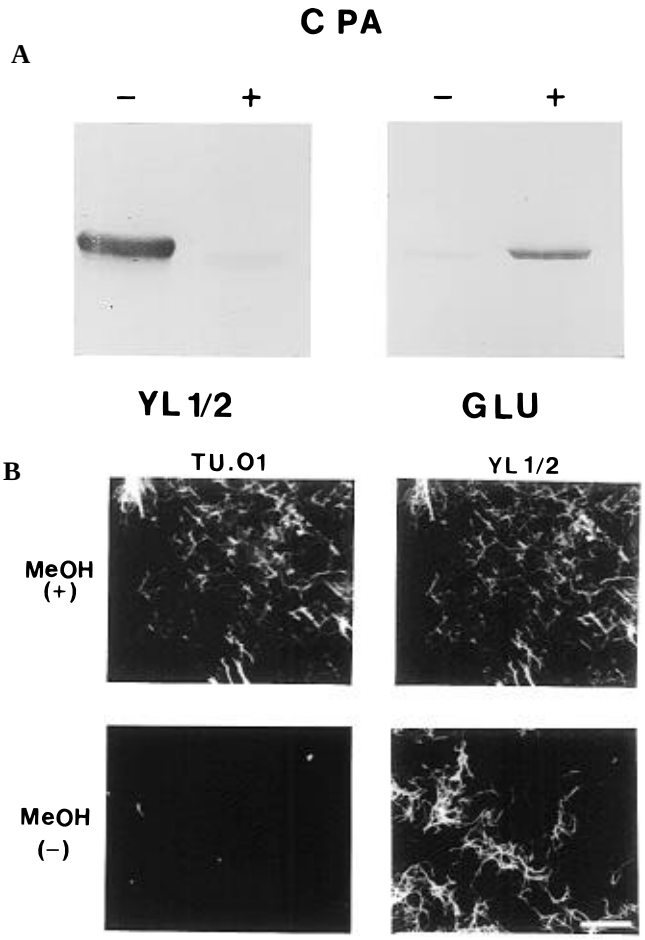

FIGURE 6: Accessibility of the carboxy-terminal end of α-tubulin in isolated A tubules. (A) Immunoblot analysis of detyrosination of A tubules in whole axonemes by carboxypeptidase A (CPA) treatment. A tubules were prepared from control axonemes (CPA^-) and carboxypeptidase A-treated axonemes (CPA^+) as described in Materials and Methods. Proteins from CPA^- and CPA^+ tubules were separated by SDS−PAGE (8% gel), transferred onto nitrocellulose, and incubated with mAb $YL^1/_2$, which reacts only with tyrosinated α-tubulin, or with Glu α-tubulin antibody, which reacts only with detyrosinated α-tubulin. Note the complete detyrosination of A tubules when axonemes are exposed to carboxypeptidase A. (B) Antibody accessibility of the carboxy-terminal end of α-tubulin in A tubules. A preparation of A tubules was double stained with mAb TU.01 and mAb $YL^1/_2$ with ($MeOH^+$) or without ($MeOH^-$) a methanol fixation step. Methanol-fixed A tubules reacted strongly with both antibodies. In the absence of methanol fixation, the tubules retained a native structure and lack reactivity with mAb TU.01 (see the text for explanation). Such native microtubules reacted normally with mAb $YL^1/_2$, which recognizes the carboxy-terminal epitope EEY in tyrosinated α-tubulin.

of flagella. The tail of dynein binds tightly to the A tubule and not to the B tubule, while the large globular heads have an ATP-dependent binding site for a B tubule (Alberts et al., 1994). We have now shown that the difference between A and B tubules extends to the tubulins themselves. They reflect, at least in sea urchin sperm axonemes, different tubulin variants, which arise by differential posttranslational processing. It remains to be seen whether these differences in tubulin variants are connected with any functional differences of A and B tubules. Finally, the A tubule with its full complement of tyrosinated α-tubulin is particularly intriguing since long-lived cytoplasmic microtubules were thought to be subject to extensive detyrosination (Schulze et al., 1987; Wehland & Weber, 1987a).

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