Mutations of Tubulin Glycation Sites Reveal Cross-talk between the C Termini of α- and β-Tubulin and Affect the Ciliary Matrix in *Tetrahymena*

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Two types of polymeric post-translational modifications of αβ-tubulin, glycation and glutamylation, occur widely in cilia and flagella. Their respective cellular functions are poorly understood. Mass spectrometry and immunoblotting showed that two closely related species, the ciliates *Tetrahymena* and *Paramecium*, have dramatically different compositions of tubulin post-translational modifications in structurally identical axonemes. Whereas the axonemal tubulin of *Paramecium* is highly glycylated and has a very low glutamylation content, the axonemal tubulin of *Tetrahymena* is glycylated and extensively glutamylated. In addition, only the α-tubulin of *Tetrahymena* undergoes deacetylation. Mutations of the known glycation sites in *Tetrahymena* tubulin affected the level of each polymeric modification type in both the mutated and nonmutated subunits, revealing cross-talk between α- and β-tubulin. Ultrastructural analyses of glycation site mutants uncovered defects in the doublet B-subfiber of axonemes and revealed an accumulation of dense material in the ciliary matrix, reminiscent of intraflagellar transport particles seen by others in *Chlamydomonas*. We propose that polyglycylation and/or polyglutamylation stabilize the B-subfiber of outer doublets and regulate the intraflagellar transport.

Microtubules are subject to a set of post-translational modifications (PTMs) whose significance has emerged only recently (1–4). Among PTMs, two polymeric modifications, glutamylation and glycation, substantially increase the heterogeneity of the αβ-tubulin heterodimer. These tubulin modifications, referred to as polyglutamylation and polyglycylation, correspond to the addition of a peptide polymer consisting of several glutamates (5) or glycines (6) onto the C-carboxyl group of a glutamate of the primary sequence of tubulin. These two PTMs will be referred to as “polymodifications” throughout this report. Polymodifications generate peptide branches of variable lengths distributed on several glutamate acceptor sites in the C-terminal tails of α- and β-tubulin (7–9). Both polymodification types are enriched in flagella and cilia of protists and metazoa cells (4) and were implicated in axoneme motility (10, 11). Whereas polyglycylation is restricted to axonemes in the ciliated and flagellated metazoan cells, polyglutamylation occurs in both axonemes and basal bodies (12–15). In ciliates, both polymodification types are not only present in cilia and basal bodies (15–18), but also occur on the more dynamic intracytoplasm microtubules (17, 19, 20). Ciliates assemble up to 17 types of distinct microtubular arrays in a single cell (19, 21–23). In these highly differentiated cells, the microtubular networks are involved in nuclear divisions, intracellular transport, organelle positioning, and are associated with specialized organelles that function in osmotic regulation, feeding, excretion, and locomotion. The α- and β-tubulins of ciliates are biochemically heterogeneous (17, 20, 24), suggesting that structural differences among tubulin isoforms are important in generating functionally distinct types of microtubules in a single cell. However, genetic and biochemical studies showed that in ciliates only one or two gene isoatypes of α- and β-tubulin form the bulk of microtubules (6, 25–28). Therefore, the large number of tubulin isoforms present in ciliates is mainly the result of PTMs (see refs. 6 and 29). Thus, ciliates provide a favorable model for assessing the function of tubulin PTMs.

Recently, by means of site-directed mutagenesis, three and five glycation sites were identified in α- and β-tubulin of *Tetrahymena*, respectively (30). Whereas all three sites of α-tubulin are dispensable, similar sites in β-tubulin are essential for cell survival. Specifically, among the five sites on β-tubulin, single and double site mutations did not affect cells, but triple site mutations resulted in either hypomorphic or lethal phenotypes, in a site-specific manner. Viable triple site β-tubulin mutants grow and move slowly and have occasional defects in cytokinesis (30). The lethal mutants have disorganized and paralyzed axonemes and are completely blocked in cytokinesis.
before their death. The axonemes in the lethal mutants lack the central pair and contain peripheral singlets instead of doublets (31).

The five glycylatable glutamates in β-tubulin of *Tetrahymena*, deduced from site-directed mutagenesis and immunoblotting (Glu437, Glu438, Glu439, Glu440, and Glu442), are homologous to the glycosylation sites identified by mass spectrometry in *Paramecium* (9). Less is known about the site location and function of glycosylation in both ciliate species. Immunological data showed the presence of glycosylated tubulin in ciliates (17). Because glycosylation and glycosylation sites are located in proximity within the C-terminal tails of α- or β-tubulin (32–34), it is possible that the mutations of glycosylation sites of *Tetrahymena* (30, 31) also affect glycosylation.

Here, a structural analysis of WT and mutated *Tetrahymena* tubulins was undertaken to systematically characterize PTMs on each tubulin subunit and to determine how mutations of the known glycosylation sites affect the level and the extent of each polypeptide type.

By mass spectrometry and immunoblotting, we show that, in contrast to axonemal tubulin of *Paramecium*, which exhibits a quantitatively minor polyglycosylation (6, 17), the *Tetrahymena* α/β-tubulin is extensively polyglycosylated. Noticeably, the mutations of the known glycosylation sites not only affected glycosylation, but also glycosylation on the same tubulin subunit. Unexpectedly, mutations of glycosylation sites on one tubulin subunit (α or β) affected the levels of polypeptides on the nonmutated subunits of the α/β-tubulin dimers. Thus, cross-talk between α- and β-tubulin exists, which affects the total levels of polypeptides on microtubules. Our ultrastructural analyses of cilia of the viable polypeptide mutants showed that the β-subfiber of the outer doublets (ODs) is strongly affected. Furthermore, the glycosylation site mutants accumulate electron-dense aggregates, similar to the in-fragellar transport (IFT) particles of *Chlamydomonas*, suggesting that tubulin polypeptides are involved in IFT.

**MATERIALS AND METHODS**

**Cell Culture—** *Paramecium tetraurelia* and *Tetrahymena thermophila* strains were grown as described previously (20, 29).

*Tetrahymena Mutants—** Site-directed mutagenesis of the major α-tubulin of *Tetrahymena* encoded by the ATU1 gene (Atu1p) and the major β-tubulin encoded by the BTU1 gene (Btu1p) was performed as described before (30). In *T. thermophila*, the wild type (WT) sequence of C-terminal tail domain is437IETAEGEGEY449, with the known polyglycosylation sites underlined. In the present work, viable β-tubulin mutants with a tripeptide substitution of adjacent glutamates (E) by aspartate (D) (30) or alanine (A) residues were used. The tail sequences of these mutants are427DATAEE-FEEDDDG439 (the mutant named 3βEDD40) and427DATAEE-EFEEAAAG445 (βEAAA45). A quadruple glycosylation site β-tubulin mutant with the tail sequence427DATAEEEGEFDGDDG439 (3βEDDG43) was also used. For α-tubulin, the WT C-terminal tail sequence is437IETAEGEEEGY449, with the known polyglycosylation sites underlined, whereas the tail sequence of the viable mutant used here is437IETAEGEGAAGY449 (AAA450) (30).

**Preparation of Axonemal Tubulin—** The axonemal tubulin of *Paramecium* was extracted from cilia as described previously (35). WT and mutant *Tetrahymena* cilia were prepared as described (30), and axonemal tubulin was extracted exactly as done for *Paramecium*.

**Protein Electrotophoresis and Immunoblotting—** Axonemal proteins were separated by SDS-PAGE on 10% polyacrylamide mini-gels (36), containing 0.1% (wt/wt) SDS (99% pure, BDH, Poole, UK) at pH 8.3, according to Suprenant et al. (24). Under these conditions, the *Paramecium* or *Tetrahymena* α-tubulin migrates faster than β-tubulin. Proteins were transferred onto nitrocellulose by the method of Kyhe-Andersen (37). The blots were stained with Ponceau red, washed in antibody buffer, and then incubated overnight with one of the following primary antibodies: TAP 952 or AXO 49 monoclonal antibodies (mAbs), raised against *Paramecium* axonemal tubulin (38) and directed against mono- and polyglycosylated tubulins, respectively (20). GT335 mAb directed against glycosylated tubulin (39); TAP 9311 (38) or DM1A (40; purchased from Amersham Biosciences) mAbs, directed against N- and C-terminal sequences of α-tubulin, respectively; C140 (41) polyclonal antibodies directed against a N-terminal sequence of β-tubulin. After extensive washing, blots were incubated with peroxidase-labeled sheep anti-mouse and donkey anti-rabbit IgG antibodies and processed for enhanced chemiluminescence (ECL) (Amersham Biosciences). The GT335 and C140 antibodies were kindly provided by Dr. F. Denoulet (University of Paris VI, France) and Dr. J. M. Andreu (Centro de Investigaciones Biologicas, Madrid, Spain), respectively.

**Protocolic Digestion of Axonemal Tubulins—** Axonemal tubulin, typically 0.2–0.5 mg, was digested with the endoprotease Asp-N (sequencing grade protease, Roche Applied Science) at an enzyme to tubulin protein ratio of 1:400 (w/w) in 50 mM Tris-HCl, pH 8.5, at 37 °C for 6 h. After digestion, the peptide mixture was frozen at −20 °C. Prior to chromatography, the peptide sample was briefly sonicated and clarified by centrifugation at 12,000 × g for 2 min.

**Purification of C-terminal Tubulin Peptides—** The endoprotease Asp-N digestion of *Tetrahymena* tubulin produces the C-terminal peptides of α- and β-tubulin beginning with an aspartate at positions 431 and 427, respectively, when the digestion is complete. Following incomplete digestion, longer α-tubulin C-terminal peptides were generated, beginning with an aspartate at position 424. The C-terminal peptides were separated on an arginine-Sepharose column and either desalted on a C18 Sep-Pak cartridge prior to direct analysis of the total peptide pools, as previously described (7), or further separated by reverse-phase HPLC. In the latter case, about 100 μg of digested tubulin was injected into a C18 reverse-phase HPLC column (5 μm, 250 × 1 mm, Hypersil BDS-C18, flow rate of 200 μl/min). Peptides were eluted with a gradient of solvent A (0.1% trifluoroacetic acid) and solvent B (80% acetonitrile, 0.1% trifluoroacetic acid) consisting of 1% solvent B for 10 min, 1% to 100% solvent B in 1 min, followed by 10 min solvent B. The mass of the peptide pool is 337,000 Da. The mass spectrometer was operated in 2,5-dimethoxy-4-hydroxicinnamic acid (3,5-dimethoxy-4-hydroxicinnamic acid, Aldrich) in 30% acetonitrile, 5% solvent B in 1 min, 5% to 100% solvent B in 10 min. Peptide elution was monitored at 214 nm.

**MALDI-TOF Mass Spectrometry—** Mass spectra were acquired in the linear mode on a MALDI-TOF mass spectrometer (Voyager-STR, Perceptive Biosystems, Inc., Framingham, MA) equipped with a delayed extraction device. Desorption was produced by a nitrogen laser beam (λ = 337 nm). Delayed extraction time was set at 200 ns. The sample was mixed 1:1 (v/v) with a saturated solution of either sinapinic acid (3,5-dimethoxy-4-hydroxicinnamic acid, Aldrich) in 30% acetonitrile, 0.1% aqueous trifluoroacetic acid or 2,5-dihydroxybenzoic acid (Aldrich) in 0.1% aqueous trifluoroacetic acid, and analyzed in the negative or positive ion mode, respectively. Except when indicated, the mass spectra were formed using a mixture of neurotensin, adrenocorticotropic hormone (residues 18–39) and adrenocorticotropic hormone (residues 7–38) with average m/z of 1671.95, 2464.71, and 3658.17, respectively, for the deprotonated ions in the negative ion mode, and of 1673.95, 2466.71, and 3660.17 for the protonated ions.

**Transmission Electron Microscopy—** Cells were washed with 10 mM Tris, pH 7.4, and fixed in 1% glutaraldehyde in 50 mM cacodylate buffer, pH 7.4, at room temperature for 1 h, washed three times in cacodylate buffer, and postfixed in 1% osmium tetroxide in cacodylate buffer for 1 h at room temperature. After three washes in cacodylate buffer, the cells were embedded in 2.5% agar and dehydrated before inclusion in Epon. Thin sections were obtained with a Leica ultramicrotome and contrasted with uranyl acetate and lead citrate. Sections were observed with a Philips 208 electron microscope.
Tubulin Post-translational Modifications and Cilia Integrity

RESULTS

Post-translational Modifications of Axonemal Tubulins of Paramecium and Tetrahymena—We examined the status of post-translational modifications of the C-terminal peptides of axonemal tubulin extracted from WT Tetrahymena and compared the Tetrahymena data with those of the already extensively characterized axonemal tubulin of Paramecium.

Western blots with TAP 952 and AXO 49 mAbs, which recognize mono- and polyglycylated tubulins, respectively, showed that the axonemal tubulins of both ciliates contain mono- and polyglycylated sites in both subunits (Fig. 1). Note that, under conditions described under “Materials and Methods,” ciliate α-tubulins migrate faster than β-tubulins (24). Noticeably, a striking difference in reactivity with the anti-glutamylated tubulin mAb, GT335, indicated the presence of a higher level of glutamylation in Tetrahymena than in Paramecium (Fig. 2).

We purified the C-terminal tubulin peptides from cilia of both species by arginine-Sepharose chromatography (7), following the endoproteinase Asp-N digestion. An analysis of the resulting total peptide pools by MALDI-TOF mass spectrometry gave a broad picture of the modifications of both α- and β-tubulin C-terminal peptides (Fig. 3). This approach shows the diversity and reveals the most abundant peptide variants. Fig. 3 compares the C-terminal peptide pools of Paramecium (panels a and b) and Tetrahymena (panels c and d) axonemal tubulins. Using Asp-N, β-tubulins from both Paramecium and Tetrahymena were cleaved at the N-terminal side of Asp427, whereas α-tubulin was cleaved mainly at Asp424, but also less often at Asp431, generating “long” and “short” α peptides, respectively.

In the Paramecium spectrum (Fig. 3a), the predominant β peptide (experimental average m/z = 2139.42) is a hexaglycylated variant (calculated average m/z = 2140.03). The other ions observed in this mass spectrum form a series with differences of 57 Da, the mass of a single glycine residue. The range of masses of these isoforms indicates addition of 4–18 glycines. In the spectrum shown in Fig. 3b, the long peptides of α-tubulin were observed. The two predominant peptides of isotypes α1 and α2 are triglycylated, and additions of 1–19 glycines were detected. A similar composition of the glycylated
Table I compares the types of polymodifications detected in WT Paramecium and Tetrahymena in peptide pools and HPLC fractions. It appears that, in Paramecium, both β- and α-tubulin subunits are glycylated to a larger extent than in Tetrahymena. The most striking difference involves polyglutamation. Whereas this modification was not detected in Paramecium by MS, even in minor HPLC fractions, glutamylation is the most abundant modification of the Tetrahymena α-tubulin and can be detected in HPLC fractions of the β-tubulin subunit as well.

Composition of Post-translational Modifications in the Axonemal Tubulin from Glycylation Site Mutants of Tetrahymena—

The structural approach described above was used to analyze the post-translational modifications, and particularly the polymodifications, in the tubulin C-terminal peptides of Tetrahymena.
Thus, despite an overall decrease in the extent of glycylation of isoforms in the region of mutant "Materials and Methods"), the AXO 49 mAb revealed a smear of slowly-migrating peptides comprising one to seven additional glutamates by aspartates or alanines in α-tubulin (βEDDD440 and βEAAA440). Like the βEDDD440 mutant (30), βEAAA440 grew and swam more slowly than the WT and underwent infrequent arrests in cytokinesis. We also analyzed a triple mutant of α-tubulin (αAAA447), which previously was found to have a normal phenotype (30).

Western blot analyses of WT axonemal tubulin showed that the AXO 49 mAb reacts more strongly with the polyglycylated epitopes on β-tubulin than on α-tubulin (Fig. 5). In βEDDD440, the reactivity with the β-tubulin region was much weaker compared with WT. This is consistent with the previous quantitative immunoblotting analysis, which showed that the level of AXO 49 epitopes in βEDDD440 β-tubulin was only 24% of WT, and supported the conclusion that sites of glycylation were eliminated in this mutant (30). However, under the present improved gel electrophoresis conditions (see "Materials and Methods"), the AXO 49 mAb revealed a smear of slowly-migrating isoforms in the region of mutant β-tubulin, contrasting with the WT β-tubulin which appeared as a sharp band (Fig. 5). Note that in previous studies a correlation has been established between the presence of a smear of AXO 49 reactive isoforms and the presence of hyperglycylated variants (e.g. refs 10, 20). Thus, despite an overall decrease in the extent of glycylation of β-tubulin in the βEDDD440 mutant, a subset of isoforms may contain abnormally long chains of polyglycine. This conclusion was subsequently confirmed using MS (see below). In βEAAA440, both tubulin subunits were only faintly detected (Fig. 5), indicating the presence of a lower extent of polyglycylated compared with the WT and βEDDD440. In αAAA447, previous results showed an AXO 49 reactivity with the β-tubulin band only, leading to the conclusion that in this mutant all glycylation sites on α-tubulin were eliminated (30). Western blots with GT335 showed that axonemal tubulins of βEDDD440 (Fig. 2), βEAAA440 and αAAA447 (not shown) were less reactive compared with WT, indicating that all these mutations affect not only glycylation but also glutamylation.

Post-translational modifications of axonemal tubulin of the βEDDD440 mutant were further characterized by mass spectrometry. In the first step, the pools of β- and α-tubulin C-terminal peptides were analyzed by MALDI-TOF MS (Fig. 6). The mass spectrum of the β-tubulin peptides from the βEDDD440 mutant (Fig. 6a) was complex. The first molecular ion (experimental average \( m/z = 1871.16 \)) was identified as a nonmodified β-tubulin peptide starting at residue Asp\(^{327} \) (calculated average \( m/z = 1871.02 \)). The prominent series of ions correspond to glycylated forms with one to seven glycine residues added. The monoglycylated form represents the predominant peptide of this series and of the entire spectrum. A second series of ions corresponding to monoglutamylation β-tubulin peptides was observed. The latter ions were detected in a monoglutamyalted form, which represents the most abundant ion of this series, and in bi-, tri-, and tetraglycylated form. An additional less abundant C-terminal peptid of βEDDD440 β-tubulin was both biglutamylated and monoglycylated. Thus, the MS analysis of peptide pools of βEDDD440 β-tubulin shows that: (i) the predominant isoform in the mutant is monoglycylated, whereas it is tetraglycylated in WT; (ii) glutamylated β-tubulin is present in the mutant, whereas it was not detected in similar pools of WT. This lack of detection in WT could be explained by the difficulty of some peptides to desorb from the matrix when the overall heterogeneity of the peptide mixture is very high. This ion suppression observed for some less abundant peptides present in a complex mixture has been reported as spectral suppression (42). The sensitivity of detection is usually increased for less heterogeneous peptide mixtures. This explains the detection of low abundance of peptides in HPLC-purified fractions, as is in fact the case for glutamyalted WT β-tubulin peptides comprising one to seven additional glutamates (Table I).

In the mass spectrum of α-tubulin peptides from the...
βEDDD440 mutant (Fig. 6b), a unique series of ions was observed. It begins with the predominant ion (experimental average \( m/z = 2682.13 \)) corresponding to a detyrosinated, non-glycylated, and nonglutamylated α-tubulin peptide starting at residue Asp424 (calculated average \( m/z = 2681.77 \)). The following ions in the same series correspond to the addition of 1–26 glycine residues. Even though the number of added glycine residues is considerably higher compared with WT, the nonmodified peptide is still predominant. The most striking difference with the WT is the absence of glutamylated α-tubulin peptides, whereas this modification is predominant in the WT.

The βEDDD440 tubulin peptides were also subjected to HPLC separation prior to MALDI-TOF MS. As shown in Fig. 7, for two HPLC fractions analyzed, high levels of glycylation were observed for both β- (Fig. 7a) and α-tubulin (Fig. 7b) peptides with the detection of up to 42 and 37 additional glycine residues, respectively (Table I). These results are in agreement with the detection of a smear of AXO 49 immunoreactive tubulin isoforms in βEDDD440 (see Fig. 5), indicating the presence of highly modified isoforms. Such hyperglycylated isoforms were not observed in HPLC fractions of WT tubulin peptides (Fig. 4). Thus, in addition to the overall decrease in glycylation on β-tubulin, hyperglycylolation affects subsets of β- and α-tubulin isoforms in the βEDDD440 mutant.

In conclusion, for the βEDDD440 mutant, the most intriguing result lies in the composition of the nonmutated α-tubulin. Whereas the predominant modification in WT α-tubulin is glutamylation, this modification was not detected in the βEDDD440 α-tubulin, even after HPLC purification. Furthermore, the maximal number of added glycine residues increases considerably in βEDDD440 α-tubulin. These data strongly suggest that mutations of polymodification sites in the C-terminal tail of β-tubulin induce extensive changes in the composition of PTMs in the nonmutated α-tubulin subunit.

To examine further the phenomenon of cross-talk between the α- and β-tubulin subunits, we analyzed additional Tetrahymena mutants. First, another β-tubulin mutant, βEAAA440, was examined, in which the same three modification sites (mutated to aspartates in the βEDDD440 mutant) were substituted by alanines.

The mass spectrum of the α-tubulin C-terminal peptides of the βEAAA440 mutant (Fig. 8a) showed a series of ions with mass increments of 57 Da, the mass of one glycine residue. The first ion of the series, which is also the predominant ion of the mass spectrum (experimental average \( m/z = 2684.68 \)) corresponds to a C-terminal detyrosinated and nonpolymodified α-tubulin peptide starting at residue Asp424 (calculated average \( m/z = 2683.78 \)). The additional ions correspond to the presence of one to eight glycine residues. Thus, the maximal number of glycine residues is considerably lower compared with βEDDD440 α-tubulin. As in the case of the βEDDD440 mutant, we did not detect any glutamylation on the α-tubulin subunit, confirming the general influence of the β-tubulin mutations on the composition of polymodifications in the nonmutated α-tubulin.

To determine whether the influence of the polymodification site status on the nonmutated subunit is reciprocal, we examined β-tubulin of a mutant of α-tubulin glycylation sites, αAAA447. Previous study showed that the glycylation sites on α-tubulin are not essential and their absence does not change the gross phenotype (30). The mass spectrum of β-tubulin C-terminal peptides of the αAAA447 tubulin mutant (Fig. 8b) also contained a series of ions with mass increments of 57 Da. The predominant β-tubulin C-terminal peptide (experimental average \( m/z = 2142.62 \)) was a tetraglycylated form (calculated average \( m/z = 2143.02 \)), and the whole series of glycylated isoforms reflects additions of 2–26 glycine residues. The maximal number of additional glycine residues in the β-tubulin subunit is therefore higher in the αAAA447 mutant than in WT. Glutamylation was not detected in the peptide pool on β-tubulin of the αAAA447 mutant. Because glutamylation appears to be quantitatively minor also on WT β-tubulin, an effect of the αAAA447 mutation on glutamylation of the nonmutated β subunit could be below the limit of detection. Therefore, the only noticeable effect observed following the mutation of polymodification sites in the C-terminal tail of α-tubulin is an increase in the maximal glycylation level of the nonmutated β-tubulin subunit.

Ultrastuctural Analysis of Cilia in Glycylation Site Mutant Cells—Previous studies showed severe defects in the structure of axonemes in the nonviable β-tubulin mutant (βBDDE440), including lack of central pair and conversions of peripheral doublets into singlets (31). The viable hypomorphic mutants in which we analyzed the composition of modifications by MS (above) display slow motility and growth (30), but the ultrastructure of their cilia has not been studied. Because we observed that the βEAAA440 mutant displays a more significant loss of polymodifications compared with βEDDD440, we further tested whether any structural changes could be detected in cilia of viable mutants and whether the extent of these changes correlates with the extent of loss of polymodifications.

A transmission electron microscopy examination of thin sections revealed a class of cilia with profound defects in the axonomical doublets. In a subpopulation of cilia from βEDDD440 and βEAAA440 mutants, cross-sections of the axonemes showed...
that one or more B-tubules among the nine peripheral doublets were opened (Fig. 9, a, b, c, and e), indicating lack of portions of microtubule protofilaments or lack of proper attachment of the B-subfiber edge to the A-tubule. The proportion of such cilia was higher in /H9252 EAAA440 mutants than in /H9252 EDDD440, and such defects were rarely encountered in the /H9251 AAA447 mutant (Table II). Thus, the higher incidence of axonemal defects correlates with decreased glycylation and glutamylation on /H9252-tubulin in /H9252 EAAA440 compared with /H9252 EDDD440. In contrast, the low incidence of defects in the /H9251 AAA447 mutant is consistent with a nonessential role of glycylation sites on /H9251-tubulin (30). Furthermore, many /H9252 EDDD440 and /H9252 EAAA440 cilia displayed an electron-dense material in the space between the outer doublets and the ciliary membrane (Fig. 9, e and f) or were completely filled with this dense material (Fig. 9e, arrowhead). Such changes were rarely seen in WT or /H9251 AAA447 cilia. In the affected mutant cilia, an examination of the B-tubule showed a microtubule wall still detectable but with a center often filled with dense material; a single central microtubule is visible. In f, most of the B-tubules are not visible. Scale bar, 0.1 μm.

To address the possibility that the higher frequency of defects in /H9252 EAAA440, compared with /H9252 EDDD440, was induced by the presence of alanine instead of aspartate residues in the primary sequence of β-tubulin, we analyzed another /H9252-tubulin mutant where four glycylation sites (instead of three) were substituted by aspartate residues (/H9252 EDDDGD442). Like the triple site /H9252-tubulin mutants, the latter one grew more slowly than the WT; it displayed cytokinesis defects similar to those filled with the electron-opaque structures was also higher in /H9252 EAAA440 than in /H9252 EDDD440 and /H9251 AAA447 mutants (Table II).

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**Table II**

*Morphology of cilia in WT and mutant Tetrahymena*

|        | WT  | βEDDD440 | βEAAA440 | αAAA447 |
|--------|-----|----------|----------|---------|
| n (transverse sections) | 37  | 37       | 28       | 32      |
| Normal axonemes          | 34  | 24       | 13       | 31      |
| Axonemes with opened      | 3   | 13       | 15       | 1       |
| B-tubules or with    |     |          |          |         |
| peripheral singlets      |     |          |          |         |
| n (transverse and oblique| 881 | 496      | 429      | 1131    |
| sections)                |     |          |          |         |
| Cilia with opaque structures | 5  | 43       | 14       |         |

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**FIG. 8.** Mass spectra of the C-terminal peptides of α-tubulin from βEAAA440 Tetrahymena mutant (a) and of β-tubulin from αAAA447 Tetrahymena mutant (b). Peptides were analyzed in positive ion mode.

**FIG. 9.** Ultrastructural analysis of cross-sections of WT and mutant Tetrahymena cilia. a–c, βEDDD440 mutant cilia show an opening of the B-tubule (arrows). d, WT. e and f, βEAAA440 mutant cilia show an electron-dense material in a ring between peripheral ODs and the membrane. In e, opened B-tubules are visible (arrows), with their center filled with dense material; a single central microtubule is visible. In f, most of the B-tubules are not visible. Scale bar, 0.1 μm.
peripheral microtubules are visible. In arrow the basive dense material. In central microtubule (Fig. 10 a). In one of these cilia, one arrow shows bridges connecting the B-tubule to the membrane. In d, numerous oral cilia contain the opaque structures between several ODs and the membrane (arrows). In one of these cilia, one central microtubule is missing (arrow). In one of these cilia, one arrowhead with no microtubule visible (arrowhead). Scale bar, 0.1 μm.

DISCUSSION

Different Compositions of Tubulin Post-translational Modifications in Tetrahymena and Paramecium Contribute to Assembly of Structurally Identical 9+2 Axonemes— We have previously shown that, in Paramecium, axonemal tubulin is highly glycylated, whereas glutamylation represents a quantitatively minor PTM, given that it was only detected by immunoblotting and not by mass spectrometry (6, 17). The present study revealed that the Tetrahymena axonemal tubulin is glutamylated at much higher level compared with Paramecium. The α-tubulin subunit in Tetrahymena appears to be more glutamylated than the β subunit. In contrast to glutamylation, the level of glycylation was found to be higher in β than in α-tubulin. This indicates that in Tetrahymena the dominant substrate for polyglycylation is the β subunit, whereas the α subunit is preferred for polyglutamylation. This is in contrast to Paramecium where polyglycylation affects similarly both tubulin subunits. In addition, the entire axonemal α/β-tubulin of Paramecium is glycylated, whereas nonmodified α-tubulin was readily detected in Tetrahymena.

In Tetrahymena, a substantial portion of tubulin isoforms, especially in the α subunit, is both glutamylated and glycylated on the same molecule, and each level of glutamylation coexists with one or more levels of glycylation. The coexistence of both polymodifications in a single molecule has also been found in Giardia lamblia and in the axonemes of sea urchin and bull sperm (32–34), but in those species such isoforms appeared to be only minor variants. Given the presence of numerous glutamates within the primary sequence of the C-terminal tail of α- and β-tubulin (six and nine, respectively), the array of poly-
modified variants could be generated by the presence of closely juxtaposed or even shared polyglutamylation and polglycylation sites. Alternatively, a single site could bear lateral chains combining glutamate and glycine residues, as previously suggested (32).

Another striking difference between the two ciliate species is the unique presence of detyrosination of α-tubulin in Tetrahymena. In Paramecium, the α-tubulin genes all lack the C-terminal tyrosine codon entirely (28), and the MS analyses did not reveal any proteolytic event at the C terminus (6). In contrast, the majority of α-tubulin peptides in Tetrahymena was found to be detyrosinated. A novel type of α-tubulin carbboxypeptidase is likely involved in Tetrahymena, because the penultimate amino acid in the α-tubulin is glycine and not glutamate as in most other species.

The differences between Tetrahymena and Paramecium were unexpected, given that both ciliates belong to the same phylogenetic class known as Oligohymenophorea (45) and have a similar cytoskeleton design (46). The axonemes of these ciliates appear to be identical based on electron microscopy. Thus, different combinations of PTMs in various organisms may contribute to assembly of essentially identical structures. Furthermore, some species appear to lack entirely one of the polymodification types; for example, in Trypanosoma, which assembles both axonemes and complex pellicular microtubules, tubulin is glutamylated but lacks glycylation (47). In contrast, in Paramecium, glycylation is quantitatively minor. It appears therefore that the two polymodification types, despite their distinct chemical structure, are involved in similar functions and could cooperate (as suggested for mammalian sperm motility, see Ref. 13) or even substitute for each other. Growing evidence indicates that PTMs act on microtubules by modulating their affinities for interactors such as microtubule-associated proteins (MAPs) and molecular motors (see below). In distinct species, PTMs and interactors that are dependent on PTMs could co-evolve while maintaining a similar structural design of microtubular organelles.

Cross-talk between Polymodification Sites in α- and β-Tubulin Subunits—We found that mutations of three of the five glycylation sites in Tetrahymena β-tubulin change glycylation and glytubulinization levels of axonemal tubulin on both the mutated and nonmutated tubulin subunits.

The βEDDD⁴⁴⁰ tubulin displayed a decrease of reactivity with GT335 (Fig. 2), whereas a decrease of the number of additional glycines was observed in the most abundant β-tubulin isoform (monoglycylated in βEDDD⁴⁴⁰ instead of tetraglycylated in the WT, Table I). This decrease of glycylation in the predominant isoform is in accordance with the fact that only two of the five glycylation sites remain in the βEDDD⁴⁴⁰ mutant. However, we also detected quantitatively minor hyperglycylated β-tubulin peptides in the same mutant. Two mechanistic explanations for this complex effect can be proposed. First, the elimination of sites could allow for addition of more glycines to the remaining sites, due to decreased competition for the glycylation enzyme. Second, the elimination of adjacent sites may increase the processivity of the glycylation enzyme, in accordance with the detection of hyperglycylated short β peptides, \( \text{EAAA}_\text{WT} \tag{47} \), containing Glu\(_\text{437} \) as a sole remaining known glycylation site. Regardless of the mechanism of hyperglycylation, our data suggest that the multiple sites of glycylation on β-tubulin influence each other.

Unexpectedly, mutations of β-tubulin glycylation sites affected dramatically both polymodification in α-tubulin. Glutamylation, which is the most abundant PTM in WT α-tubulin, could not be detected in the βEDDD⁴⁴⁰ α-tubulin by mass spectrometry. In contrast, the maximal number of added glycines, which reaches 10 in WT α-tubulin, is considerably increased (up to 37) in the βEDDD⁴⁴⁰ α-tubulin.

The strong effect of mutations on the nonmutated subunit suggests the existence of cross-talk between the two subunits of the tubulin dimers. This cross-talk could either take place within the same dimer or between two subunits of adjacent tubulin dimers within a microtubule, given that both tubulin and microtubules could be substrates of both polymodification enzymes, as shown for polyglutamylation (48). A simple model could assign to the β-tubulin subunit a role in the initial binding of enzymes responsible for deposition of polymodifications on α-tubulin. Specifically, the tubulin glutamylase could have β-tubulin as a binding site for glutamylating the α subunit, and the mutation of the tail domain of β-tubulin could affect the enzyme binding either directly, or through a conformational change in β-tubulin. There could also be a requirement of a certain level of glycylation on the α-tubulin tail for the binding of the glutamylase. It is highly relevant that the best characterized tubulin modification enzyme, the tubulin-tyrosine ligase, forms a tight complex with α/β-tubulin, involving a binding site on β-tubulin in addition to its catalytic target, the C-terminal end of α-tubulin (49). It is therefore possible that all tubulin-amino acid ligases (tubulin-tyrosine ligase and the still unknown glyyclases and glutamylases) bind to β-tubulin while they modify the tails of α- or α/β-tubulin.

A comparison of βEDDD⁴⁴⁰ and βEAAA⁴⁴⁰ mutants strengthens the idea that the β subunit is critical for the polymodifications on both β- and α-tubulin. The overall polyglycylation extent on α- and β-tubulin of βEAAA⁴⁴⁰ was much lower than that of βEDDD⁴⁴⁰ (Fig. 5 and Table I). In the βEAAA⁴⁴⁰ mutant, the presence of alanine instead of glutamate or aspartate residues in β-tubulin could decrease the affinity of the modifying enzyme(s). This could be due to the net charge decrease or to a conformational change in β-tubulin.

It is important to note that we also observed a reciprocal influence of the α subunit on β-tubulin, but it was more subtle. A mutation of the α-tubulin glycylation sites (αAAA⁴⁴⁷) increased the maximal number of added glycines to the tail of the nonmutated β subunit but did not affect the level of glycylation of the most abundant isoform (four glycines). In addition, glutamylation was not detected in the β subunit of this mutant. It is therefore possible that the binding and/or activity of the glycylation(s) and glutamylase(s) involve a cooperative interaction of tails of both tubulin subunits. Various studies suggest that the C-terminal tails are highly flexible domains at the surface of microtubules, and are able to participate in numerous interactions with tubulins and other proteins such as MAPs and motors (50–52).

Regardless of the nature of the cross-talk mechanism, our results show the existence of interdependence of polymodification sites between the two subunits of the tubulin dimers. Because the mutations of the known glycylation sites on β-tubulin also affect glutamylation, the observed phenotypic changes (Refs. 30 and 31 and this report) could represent the loss of both glycylation and glutamylation. Furthermore, the phenotype of the β-tubulin mutants may result from changes encompassing both subunits. Finally, it can not be ruled out that the mutant phenotypes resulting from mutations in one subunit are suppressed to some extent by potential compensatory changes in the mutated and nonmutated subunits (such as increased enzyme processivity leading to hyperglycylation of minor isoforms). The dissection of respective functional contributions of glutamylation and glycylation will require inactivation of genes encoding the glutamylase and glycylation enzymes.
Mutations in the Tubulin Polymodification Sites Affect the B-subfiber of Axonemal Outer Doublets and the Ciliary Matrix—Thazhath et al. (31) showed that a lethal deficiency in tubulin polyglycylation affects the structure of the axoneme, manifested by the lack of the central pair and replacement of peripheral doublets by singlets. We show here that the B-tubule of doublets is primarily affected by a mild deficiency in polymodifications. In most cases, the viable mutant axonemes have opened B-tubules. Such a defect may result from either insufficient number of protofilaments or lack of proper binding of the most lateral protofilament of the B-tubule to the A-tubule. Our analyses of the viable mutants revealed additional changes that were not obvious in the lethal mutants, including accumulation of the dense material between the doublets and the ciliary membrane, suggestive of a defect in IFT (see below).

There appears to be a correlation between the strong effect of the polymodification site mutations on the B-tubule and the distribution of polymodifications within the axoneme. Mulligner et al. (53) showed that, in sea urchin sperm flagella, the A-tubules contain unmodified tyrosinated tubulin, whereas the B-tubules contain detyrosinated tubulin that is extensively polyglycylated. A predominant occurrence of polyglutamylated tubulin in the B-tubule was shown in Spermatozopsis similis (54). Thus, it appears that polymodifications have a function either in the assembly or in the attachment of the B-tubule to the A-tubule in axonemes.

Previous data showed a potential role for polymodifications in the maturation of organelles such as cilia and basal bodies (18, 54). Polyglycylation follows a sequential mechanism, monoglycylation occurring in newly assembled microtubular structures and lengthening of polyglycine chains in mature ones (18). Accordingly, recent epitope tagging experiments showed that a lethal glycylation site mutation affects newly formed cilia as well as mature ones (55). Therefore, in Tetrahymena, polymodifications could mediate the B-tubule closure during the course of axonemal assembly or stabilize the B-tubule after the assembly. In both cases, glycylation or glutamylation could act directly or indirectly. Surprisingly, whereas long-lived microtubules in animal tissue cultured cells have been shown to be extensively post-translationally modified (56–58), the A-tubule of sea urchin sperm flagella, which is a stable microtubule, contains at least 95% of unmodified tubulin (53). Thus, a direct involvement of polymodifications in axonemal microtubule stability seems unlikely. The axoneme stabilization process could result from the doublet microtubule conformation per se (as opposed to a singlet conformation) and/or from interactions between axonemal microtubules and accessory proteins, in particular those which may be required for the doublet formation such as tektins (59, 60). The polymodifications could specifically recruit MAPs for assembly and stabilization of the B-tubule. In vitro experiments showed that polyglutamylation is involved in the interaction between tubulin and MAPs (61, 62), and a correlation between glycylation and microtubule cold stability has been established in gerbil cochlea (63), presumably involving cold MAPs (64). Our results also suggest that polymodifications affect intraflagellar transport. IFT involves the movement of large protein complexes (IFT particles) along the doublet microtubules (65–67). This process is required for flagellar and ciliary assembly as well as maintenance, due to the turnover of flagellar components (68, 69). IFT particles and flagellar or ciliary precursors are moved in the flagella or cilia by kinesin-II (44, 70, 71) and are recycled by the cytoplasmic dynein DHC1b (72). The IFT particles were shown to be preferentially associated with the B-subfiber of the outer doublet microtubules; they appear as electron-dense material located between the flagellar membrane and the peripheral doublets (43, 44, 73). Our electron microscopy observations of hypomorphic β-tubulin mutants showed cilia with an unusual electron-dense material, in the space between the ODs and the flagellar membrane, that resembles IFT particles. This material may represent gathered IFT particles, which were already shown to accumulate in mutants affected in IFT motors (44, 72–74). From MS data, βEDDD₄⁴⁰ and βEAAA₄⁴⁰ showed a decrease in the overall glutamylation levels of α/β-tubulin and in the glycylation level of the predominant isoform of β-tubulin, in comparison with the WT. Importantly, the maximal number of additional glycines analyzed in the peptide pool was lower in βEAAA₄⁴⁰ (up to eight glycines) than in βEDDD₄⁴⁰ α-tubulin (up to 26 glycines), and glutamylation was not detected in the β-tubulin peptides of the former mutant (Table I), showing that βEAAA₄⁴⁰ is more deficient in both polymodifications. This deficiency correlates with a higher proportion of cilia containing an unusual dense material adjacent to the ODs of βEAAA₄⁴⁰.

The polyglycine and polyglutamate chains are known to be distributed in a polarized fashion along the axoneme (10, 13, 54, 75, 76) and therefore could potentially act as regulators of IFT along the length of the axoneme. In addition, as observed in mammalian sperm flagella, individual doublets react differently with the anti-tubulin polymodification mAbs (12, 13, 77). A differential modification of specific ODs could provide local environments more or less favorable for the anterograde and retrograde IFT. In glycylation site mutants, the decrease in the level of polymodifications could alter the kinetics of IFT and/or composition of IFT cargoes and induce destabilization of the B-tubule and microtubules of the central pair. We also need to consider an alternative explanation, namely that the polymodifications are primarily required at the level of assembly of microtubules. The potential effect on IFT could therefore be indirect and result from the structural defects in the B-tubules, which are tracks for IFT. Nevertheless, the observation of numerous cross-sections containing the opaque structures near intact B-tubules favors the hypothesis that IFT defects precede the structural defects of the B-tubules.

The effect on IFT could be mediated by changes in the motility parameters of molecular motors. The processivity of kinesins was shown to be regulated by the interactions between conserved basic residues of the motor proteins and the acidic C termini of tubulin (78–80). The C-terminal sequence of tubulin, which interacts with kinesin (81), includes the polyglycylation and polyglutamylation sites. Accordingly, microinjection of anti-polyglutamyalted tubulin mAb (GT335) into melanophores selectively impaired kinesin-dependent pigment granule dispersion (82). The affinity of kinesin to tubulin was shown to depend on the length of the polyglutamyl chain (83), and flagellar dynein also appeared to depend on polyglutamylation and polyglutamylation for interactions with microtubules (10, 11). Polyglutamylation is already known to affect the binding of MAPs and motors to tubulins in vitro (61, 62, 83). It is conceivable that, in the Tetrahymena mutants, lack of proper levels of polymodifications disrupts the binding of these interactors. Polymodifications could specifically affect the interactions between IFT motors (kinesin-II and IFT dynein) and the axonomal microtubules and thus regulate the intraflagellar transport.

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Mutations of Tubulin Glycelylation Sites Reveal Cross-talk between the C Termini of α- and β-Tubulin and Affect the Ciliary Matrix in Tetrahymena
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