Polyglycylation of Tubulin Is Essential and Affects Cell Motility and Division in *Tetrahymena thermophila*

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Abstract. We analyzed the role of tubulin polyglycylation in *Tetrahymena thermophila* using in vivo mutagenesis and immunochemical analysis with modification-specific antibodies. Three and five polyglycylation sites were identified at glutamic acids near the COOH termini of α- and β-tubulin, respectively. Mutants lacking all polyglycylation sites on α-tubulin have normal phenotype, whereas similar sites on β-tubulin are essential. A viable mutant with three mutated sites in β-tubulin showed reduced tubulin glycylation, slow growth and motility, and defects in cytokinesis. Cells in which all five polyglycylation sites on β-tubulin were mutated were viable if they were cotransformed with an α-tubulin gene whose COOH terminus was replaced by the wild-type COOH terminus of β-tubulin. In this double mutant, β-tubulin lacked detectable polyglycylation, while the α-β tubulin chimera was hyperglycylated compared with α-tubulin in wild-type cells. Thus, the essential function of polyglycylation of the COOH terminus of β-tubulin can be transferred to α-tubulin, indicating it is the total amount of polyglycylation on both α- and β-tubulin that is essential for survival.

Key words: motor proteins • microtubules • cilia • cytoskeleton • motility

Introduction

Microtubules (MTs)[1] are ubiquitous eukaryotic cytoskeletal filaments consisting of linear polymers of noncovalently linked heterodimers of α- and β-tubulin. They form the structural framework of organelles that are essential for maintaining shape, and play major roles in cell movement, intracellular transport, and cell division. Different MT systems (e.g., cytoplasmic network, axonemes, centrioles, and mitotic spindle) contain MTs that have distinct lengths, spatial arrangements, and dynamics. Little is known about the mechanisms that result in the correct location, assembly, and function of distinct microtubules in a cell.

It seems reasonable to expect that the functional diversity of microtubules reflects underlying compositional differences among them. However, most cell types do not express enough tubulin isotypes to account for all microtubule diversity (Silflow, 1991). Ciliates, in particular, have a small number of tubulin isotypes while exhibiting diversity of microtubules comparable to that found in an entire multicellular organism (Conzelmann and Helftenbein, 1987; Gaertig et al., 1993).

In most eukaryotes, posttranslational tubulin modifications (PTMs; for reviews see MacRae, 1997; Luduena, 1998) also produce heterogeneity among α- and β-tubulins. These modifications include the following: α-tubulin acetylation (L'Hernault and Rosenbaum, 1985), removal of the COOH-terminal tyrosine of α-tubulin (Argarana et al., 1978), phosphorylation of β-tubulin (Eipper, 1972), palmitoylation of α-tubulin (Caron, 1997), polyglutamylation (Eddé et al., 1990; Alexander et al., 1991; Redeker et al., 1992; Rüdiger et al., 1992; Mary et al., 1994), and polyglycylation (Redeker et al., 1994) of α- and β-tubulin.

Polyglycylation is a recently identified PTM, so far unique to tubulin, that adds up to 34 glycine residues onto the γ-COOH groups of specific glutamate residues (Es) of α- and β-tubulin in *Paramecium* (Redeker et al., 1994). Polyglycylation occurs on several clustered Es of β-tubulin of *Paramecium* including E_{437}, E_{438}, E_{439}, and E_{441} (Vinh et al., 1999). This PTM has been found in diverse species (Rüdiger et al., 1995; Bré et al., 1996; Mary et al., 1996; Multigner et al., 1996; Weber et al., 1996), but only in cell types that have either cilia or flagella (discussed in Levilliers et al., 1995; Bré et al., 1998). mAbs specific for polyglycylated tubulins inhibited the motility of reactivated sea

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[1]*Abbreviations used in this paper*: E, glutamate residues; MT, microtubule; PTM, posttranslational modifications.

urchin spermatozoa (Bré et al., 1996), suggesting that this PTM plays a role in regulation of ciliary dynein.

We have been developing the ciliated protozoan, *Tetrahymena thermophila*, as a model for studying the in vivo function of PTMs. *Tetrahymena* and *Paramecium* belong to the same class of ciliates (Baroin-Tourancheau et al., 1992) and have similar cytoskeletal organizations (Fleury et al., 1992). *T. thermophila* maintains at least 17 distinct microtubule structures, but expresses only one type of α-tubulin, one major and one highly divergent minor β-tubulin proteins encoded by a single α- and three β-tubulin genes (Gaertig et al., 1993; McGrath et al., 1994; Li, B., and M.A. Gorovsky, unpublished results). However, *Tetrahymena* tubulins occur in multiple isoforms generated by various PTMs (Suprenant et al., 1985; Gaertig et al., 1995). The COOH termini of *Tetrahymena* α- and β-tubulin are similar to the conserved COOH termini of other axonemal tubulins, and contain several possible sites of polyglycylation. Here, we describe genetic analyses of the polyglycylatable sites of *Tetrahymena* α- and β-tubulin. We show that cells need polyglycylation sites on β-tubulin for survival, whereas similar sites on α-tubulin are dispensable. However, a lethal polyglycylation site mutation on β-tubulin could be rescued if the COOH-terminal domain of α-tubulin was replaced with the wild-type COOH terminus of β-tubulin. Thus, polyglycylation of α- and β-tubulin appears to have redundant functions and the total amount of polyglycylation on both subunits appears to be critical for cell survival.

Materials and Methods

Cell Culture

T. thermophila cells were grown in SPPA (1% proteose peptone, 0.2% glucose, 0.1% yeast extract, 0.003% EDTA·ferric sodium salt, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml fungizone) at 30°C with shaking.

Germline Transformation and Construction of BTU Double Knockout Heterokaryons

To disrupt the *T. thermophila BTU2* gene, we constructed the pTBS plasmid with the coding sequence of the 3-kb HindIII fragment of *BTU2* (Gaertig et al., 1993) replaced by the blasticidin S (bs) resistance gene, *bsr*, (Sutoh, 1993) which is controlled by the *HHF1* gene promoter (Gaertig et al., 1994a). For disruption of *BTU1*, we used the pHAB1 plasmid containing a *BTU1* fragment whose coding sequence was replaced by the *neo* gene under control of the *HHF1* promoter. To disrupt genes in the germline micronucleus, pTBS or pHAB1 DNA was purified using the Plasmid Maxi kit (Qiagen). The pTBS plasmid was linearized with EcoRI and SalI to release the *btu2::bsr1* insert, whereas the pHAB1 plasmid was linearized with SacI and SalI to release the *btu1::neo1* insert. Biolistic germline transformation was done as previously described (Cassidy-Hanley et al., 1997), except that we used gold particles (0.6 μm; Bio-Rad Laboratories) instead of tungsten. The *btu2::bsr1* transformants were selected at 60 μg/ml blasticidin S (ICN), whereas the *btu1::neo1* transformants were selected with 120 μg/ml paromomycin (pm; Sigma Chemical Co.) in SPPA. Transformants were confirmed to be heterozygous germline knockouts as described previously (Cassidy-Hanley et al., 1997). A heterozygous clone for the *btu2::bsr1/BTU2* was crossed to a strain heterozygous for the *btu1::neo1/BTU1* gene in the micronucleus. Double heterozygotes from this cross were mated to a B*VII strain (Orias and Bruns, 1976) to obtain cells with micronuclei homozygous for both disrupted alleles. Two exconjugant clones of different mating types (DB2A and DB6B) were identified as homozygotes for both disrupted BTU genes based on the appearance of bs-r and pm-r progeny in an outcross.

Introduction of Mutated Tubulin Genes by Rescue of Mating Knockout Heterokaryons

Plasmid pTUB100E3-PvuII contains the 3.2-kb HindIII genomic fragment of the α-tubulin gene of *T. thermophila, ATU1* (Hai and Gorovsky, 1997). Derivatives of pTUB100E3-PvuII containing desired mutations were made by site-directed mutagenesis (Kunkel, 1985). The same approach was used with plasmid pBTU1 (Gaertig et al., 1993) to construct mutations of the *T. thermophila BTU1* gene encoding β-tubulin. The *ATU1* genes (linearized with HindIII) were used to rescue mating heterokaryon strains lacking functional α-tubulin genes in the micronucleus (AAKO2 and AAKO5), and transformed cells were selected with 120 μg/ml pm as previously described (Hai and Gorovsky, 1997). Introduction of mutated *BTU1* genes (linearized with XbaI and HindIII) was performed by rescue transformation of mating heterokaryon strains DB2A and DB6B, and transformed progeny were selected with 60 μg/ml of bs in SPPA.

Isolation of Tetrahymena Genomic DNA and Southern Blotting

Late log phase *Tetrahymena* cells (6–8 × 10^5 cells/ml) were starved overnight in 10 mM Tris, pH 7.5, at 30°C. Total genomic DNA was isolated from 50 ml of starved cells and Southern blots were prepared as described (Gaertig et al., 1994). Radiolabeled fragments of the 3′ flanking sequence of either *BTU1* or *BTU2* gene were used as probes.

Isolation of Cilia

A 1-liter culture of *Tetrahymena* cells (4–5 × 10^5 cells/ml) was grown in SPPA at 30°C overnight with shaking. Cells were harvested at 1,300 *g* for 5 min at 4°C and resuspended in a total volume of 15 ml of 10 mM Tris, pH 7.5. Protease inhibitors were added to a final concentration of: 0.7 mM PMSF, 3 μg/ml pepstatin, 3 μg/ml leupeptin, 30 μg/ml chymostatin, 10 μg/ml trans-epoxysuccinyl-L-leucylamido-(4guanidino)butane (E-64), and 15 μg/ml antipain. Cells were deciliated by adding 90 μl of 500 mM of dibucaine-HCl (Sigma Chemical Co.) followed by swirling. Just before all cells became nonmotile when viewed under a microscope (~2–3 min), 75 ml of ice-cold 10 mM Tris, pH 7.5, were added, and the suspension was spun at 1,900 *g* for 5 min at 4°C. The supernatant was spun again as above, and cilia were collected from the second supernatant by centrifugation at 14,000 *g* for 20 min at 4°C. Cilia were washed with 28 ml of ice-cold wash buffer (10 mM Hepes, pH 7.4, 100 mM NaCl, 4 mM $MgCl_2$, and 0.1 mM EGTA) containing protease inhibitors, centrifuged at 14,000 *g* at 4°C for 10 min, resuspended (100 mg of wet ciliary pellet /ml) in cold wash buffer containing protease inhibitors, and frozen at −80°C. Ciliary protein concentrations were determined using the BCA assay kit (Pierce Chemical Co.).

Protein Electrophoresis and Immunoblotting

For α-tubulin mutants, axonemal proteins extracted from cilia were mixed with an equal volume of 2× SDS sample buffer (Gaertig et al., 1995), boiled for 3–5 min, and run on 12% SDS–polyacrylamide gels (resolving gel at pH 8.8). Gels were blotted onto Immobilon-P PVDF membranes (Millipore Corp.) using a semi-dry transfer cell (Bio-Rad Laboratories). Blots were blocked in 3% BSA in TBS and probed with mouse primary mAbs. HRP rabbit anti–mouse IgG (1:2,000 dilution; Zymed Labs, Inc.) was used as a secondary antibody. Blots were developed with the Western blot chemiluminescence Reagent Plus kit (NEN Life Science Products). For β-tubulin mutants, ciliary extracts (25–1,000 μg) were subjected to 10% SDS-PAGE, at pH 8.25 (Suprenant et al., 1985). Proteins were electrotransferred onto 0.45-μm nitrocellulose membranes at 20 V for 2 h. The blots were removed and blocked in PBST buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, and 0.1% Tween 20) containing 5% dried milk for 1 h at room temperature. Blots were incubated for 1 h at room temperature with the primary antibodies, membranes were washed in PBST and incubated with 1:3,000 dilution of goat anti–mouse or anti–rabbit antibodies linked to HRP (Amersham) for 1 h at room temperature. Blots were processed using the ECL system (Amersham), exposed to Fuji medical X-ray films, and the resulting bands were quantified by densitometry (ImageQuant Fast scan; Molecular Dynamics). For primary antibodies, we used the following mouse mAbs: TAP 952 directed against monoglycylated tubulin sites (1:1,000 dilution); AXO 49 directed against polyglycylated tubulins (1:667 dilution); general anti–α-tubulin, DM1A (1:10,000 dilution; Amersham Pharmacia); general anti–β-tubulin,

DM1B (1:10,000 dilution; Amersham Pharmacia); or polyclonal antibodies directed against 10xG peptide, anti-polyG at 1:100 dilution.

Phenotypic Analyses

Immunofluorescent detection of microtubules in *Tetrahymena* was performed as previously described (Gaertig et al., 1995). Sets of optical sections of individual cells were obtained using a laser scanning confocal microscope (model MRC 600; Bio-Rad Laboratories Inc.) and processed to obtain complete three-dimensional reconstruction. As a primary antibody, we used mAbs AXO 49, TAP 952, anti-polyG or anti-polyE antibodies generated against 10xE peptide at 1:100 dilution.

To measure growth rates, cultures grown in 50 ml SPPA at 30°C with shaking (150 rpm) were counted at 4–5-h intervals using a Coulter counter (model ZF). To determine the rates of cell movement, cells were grown to density of 2×10^5 cells/ml. 10 μl of culture were placed on microscopic slides without coverslips and analyzed using an inverted microscope (model TE300; Nikon). Paths of moving cells were recorded for 4 s using a digital color CCD camera (model VI-470; Optronics) and IM-4000 software (version 3.46p; Image Analytics). The path lengths of moving cells was determined using the NIH Image program.

Results

α-Tubulin Polyglycylation in T. thermophila Occurs at Multiple Sites and Is Not Essential

E_{445} was originally shown to be a polyglycylation site in the α-tubulin of *Paramecium* (Redeker et al., 1994) and it is conserved in *Tetrahymena* α-tubulin (Fig. 1). In β-tubulin of *Paramecium*, polyglycylation can occur on several clustered Es downstream of the originally identified site, E_{437} (Vinh et al., 1999). Two Es also immediately follow E_{445} in *Tetrahymena* α-tubulin, and we reasoned these Es could also be polyglycylated (Fig. 1). To identify the sites, α-tubulin genes mutated at these residues were used to rescue progeny of a mating between two α-tubulin knockout heterokaryons (Hai and Gorovsky, 1997). In the diploid, transcriptionally quiescent micronuclei of these knockout heterokaryons, the coding sequences of the *ATU1* genes are replaced by the coding region of the *neo* gene, which confers resistance to paromomycin in *Tetrahymena* (Kahn et al., 1993). When the two heterokaryons conjugate, the parental macronuclei are destroyed, the new macronuclei of the progeny develop from the micronuclei and receive only the disrupted copies of the *ATU1* gene. As a result, the progeny cells die when selected with paromomycin unless they are rescued with a functional *ATU1* gene. Therefore, in the rescued progeny, the mutated version of the *ATU1* gene is the only *ATU1* gene that can be expressed. We constructed plasmids encoding mutated *Tetrahymena ATU1* genes, in which the $E_{445\text{-}447}$ codons were changed to aspartic acid (D) or alanine (A) codons. Mutant genes in which all three sites are substituted (αDDD_{447} and αAAA_{447}) rescued *ATU1* mating knockout heterokaryons.

To determine if the α-tubulin in the transformants was polyglycylated, axonemal proteins were analyzed by immunoblotting with AXO 49, an mAb primarily recognizing polyglycine chains with three or more glycine residues (Gs; Bré et al., 1996, 1998). A wild-type (WT) control derived from heterokaryons transformed by the WT gene displayed two bands of ~55 kD (Fig. 2, middle). The top band comigrates with β-tubulin, whereas the bottom band comigrates with α-tubulin (Fig. 2, bottom; Suprenant et al., 1985). The αDDD_{447} and the αAAA_{447} cells showed only one band corresponding to β-tubulin (Fig. 2, middle). The same result was obtained when immunoblots were stained with TAP 952, an mAb which primarily recognizes monoglycylated tubulin sites (Bré et al., 1996, 1998; Fig. 2, top). Thus, mutating $E_{445\text{-}447}$ eliminated polyglycylation on α-tubulin, indicating that this modification is not essential. Mutant *ATU1* genes with single and double substitutions of $E_{445\text{-}447}$ in all combinations also rescued mating heterokaryons but did not abolish α-tubulin glycylation, indicating that each of the three Es serves as a modification site (data not shown).

Mutant (αAAA_{447} and αDDD_{447}) and WT cells are similar in appearance using light microscopy. No differences in growth rates were observed between the mutants and WT cells (Fig. 6 A, left). The mutant cells were indistinguishable from WT cells in rates of swimming and cilia regeneration (data not shown). We used AXO 49 and TAP 952 mAbs to examine the cellular distribution of polyglycylated tubulins in WT and mutant (αAAA_{447}) cells. In interphase WT cells, AXO 49 mAbs reacted predominantly with somatic and oral cilia (Fig. 3 B). TAP 952 antibodies reacted with somatic cilia, cortical microtubules, and intracytoplasmic microtubules during the entire cell cycle (Fig. 3 D), as well as with micronuclear spindle microtubules during division (data not shown). Within cilia, TAP 952 reacted strongly with the ciliary tips (Fig. 3 D). In contrast to

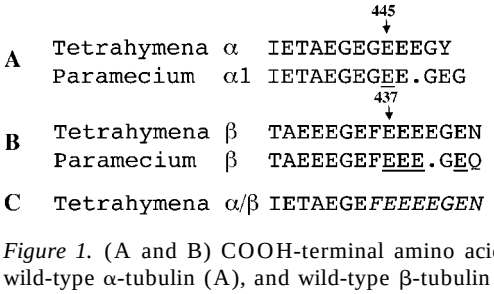

Figure 1. (A and B) COOH-terminal amino acid sequences of wild-type α-tubulin (A), and wild-type β-tubulin (B) of *T. thermophila* (Gaertig et al., 1993; McGrath et al., 1994) and *P. tetraurelia* (Dupuis, 1992; Dupuis-Williams et al., 1996). Sites known to be polyglycylated in *Paramecium* are underlined. (C) COOH-terminal sequence of the mutant α-tubulin of *Tetrahymena* in which the COOH-terminal end (positions 444–449) was replaced with the corresponding end of β-tubulin (positions 436–443).

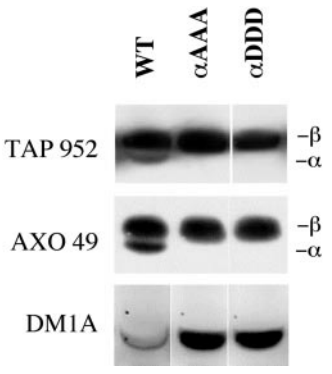

Figure 2. A Western blot analysis of axonemal proteins of *T. thermophila* WT and α-tubulin mutant strains probed with mAbs TAP 952 (top) and AXO 49 (middle) specific for glycylated tubulin and a general anti–α-tubulin mAb, DM1A (bottom).

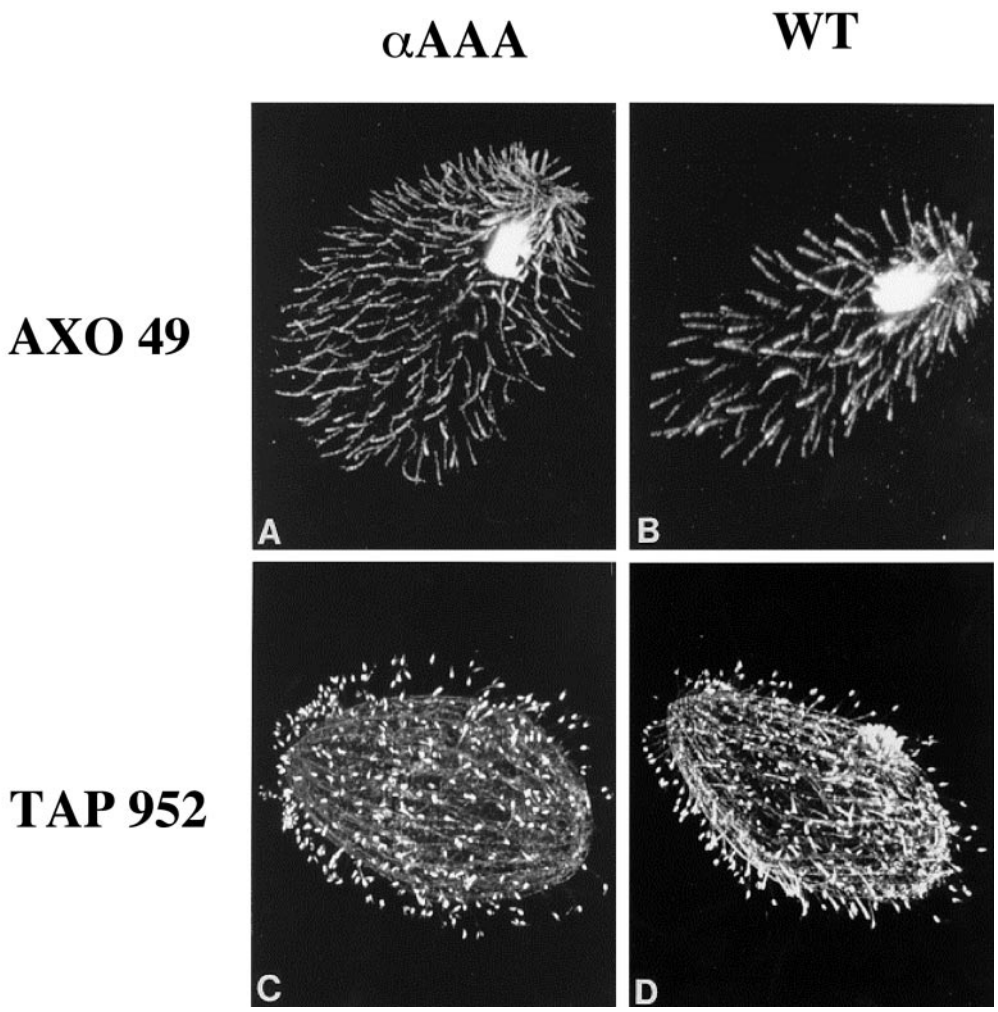

Figure 3. Projections of αAAA_{447} mutant (A and C) and WT cells (B and D) labeled with mAbs AXO 49 (A and B) and TAP 952 (C and D). Cells shown in A–C are in interphase, whereas the cell shown in D is in the stage before cell division. The oral apparatus was not labeled by TAP 952 in nondividing cells, but oral cilia were labeled in newly formed oral primordia in cells approaching cell division (D, WT cell). Similar observations were made for αAAA_{447} cells (data not shown).

AXO 49, the oral apparatus was not labeled by TAP 952 in nondividing cells, but oral cilia were labeled in newly formed oral primordia in cells approaching cell division (Fig. 3 D). The overall labeling of αAAA_{447} cells with both antibodies was indistinguishable from WT (Fig. 3, A and C). The simplest explanation is that at the light microscopic level, the pattern of α-tubulin glycylation is either the same as or is a subset of the pattern of β-tubulin glycylation. Alternatively, α-tubulin glycylation may still be restricted to certain organelles but may not be detectable by immunofluorescence because of its low level or lack of accessibility to modification-specific antibodies.

Creation of Knockout Heterokaryons for In Vivo Mutagenesis of β-Tubulin

To facilitate mutational analysis of β-tubulin, we constructed heterokaryons for transformation rescue of the β-tubulin genes. Two functionally redundant genes encode the major β-tubulin of *T. thermophila*: *BTU1* and *BTU2* (Gaertig et al., 1993; Gu, L., J. Gaertig, and M.A. Gorovsky, unpublished results). We disrupted the *BTU1* gene in the micronucleus by biolisitic bombardment using a *BTU1::neo1* fragment (Fig. 4 B). To disrupt the *BTU2* gene, we used a *btu2::bsr1* fragment in which *BTU2* contains the blasticidin S resistance gene cassette (Fig. 4 A). The two single knockout heterokaryons were used to construct two double knockout heterokaryons, DB2A and DB6B, having different mating types. When these two strains were crossed to each other and conjugation progeny were selected with bs (resistance conferred by the *BTU2::bsr1* alleles in the new macronucleus), all exconjugants died, confirming that cells require either *BTU1* or *BTU2* for survival. However, bs-resistant progeny were obtained when the DB2A and DB6A mating cells were biolistically transformed with either a *BTU1* or *BTU2* fragment (Table I and results not shown). Thus, mating double knockout BTU heterokaryon strains can be used to introduce β-tubulin genes allowing analysis of a series of mutations in residues of *Tetrahymena* β-tubulin that are homologous to polyglycylation sites identified in *Paramecium* (see next section).

Polyglycylation Sites on β-Tubulin Are Essential for Survival

E_{437} has been identified as a major site of polyglycylation on β-tubulin in *Paramecium* (Redeker et al., 1994), and three additional downstream Es are also polyglycylated (Vinh et al., 1999). A series of mutations of *BTU1* was made at the codons of the homologous sites at positions E_{437}-E_{442} of *Tetrahymena* β-tubulin (Table I). We obtained

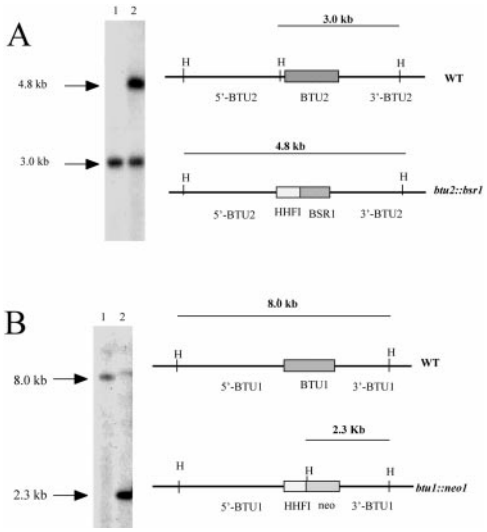

Figure. 4. (A) A Southern blot containing WT (lane 1) and *btu2::bsr1* transformant genomic DNA (lane 2) digested with HindIII restriction endonuclease. The blot was probed with an α-[^{32}P] ATP–labeled fragment containing the 3′-untranslated region of *BTU2*. In WT, the probe detects a 3.0-kb fragment of the *BTU2* gene. Gene disruption of *BTU2* using the *btu2::bsr1* fragment eliminates one HindIII site (H) and yields a fragment of 4.8 kb (see diagram on the right). The transformant DNA contains the 4.8-kb fragment and the endogenous 3-kb fragment, indicating that the transformant is heterozygous for *btu2::bsr1/BTU2*. (B) A Southern blot of total genomic DNA of WT (lane 1) and *btu1::neo1* transformant (lane 2) probed with a 3′-untranslated fragment of *BTU1*. In WT, an 8-kb HindIII fragment was detected. In the transformed cell, a smaller 2.3-kb fragment was detected consistent with gene knockout of *BTU1*, which should introduce an additional HindIII site (H, see diagram on the right).

viable, phenotypically normal progeny using fragments carrying D substitution at position 437 (βDEEE$_{440}$) or at positions 437 and 438 (βDDEE$_{440}$). No transformants were obtained for the βDDDE$_{440}$ and βDDDD$_{440}$ mutations (Table I). To test whether E$_{439}$ and E$_{440}$ are essential, we prepared a fragment with the βEEDD$_{440}$ mutation and found that it transformed at high frequency, and that surviving cells appeared normal (Table I). Thus, none of the four adjacent Es is essential by itself, but cells need to have at least two of the four consecutive Es for survival.

Table I. Sites, Transformation Efficiencies, and Gross Phenotypes of Wild Type and Various Mutations on β-Tubulin

Name of variant BTU1 gene	Amino acids (positions 437–442)	No. of transformants/μg DNA	Gross phenotype
WT	E-E-E-E-G-E	65	normal
βDEEE$_{440}$	D-E-E-E-G-E	77	normal
βEEDE$_{440}$	E-E-D-E-G-E	89	normal
βEEED$_{440}$	E-E-E-D-G-E	120	normal
BDDEE$_{440}$	D-D-E-E-G-E	141	normal
βEEDD$_{440}$	E-E-D-D-G-E	97	normal
βEDDD$_{440}$	E-D-D-D-G-E	68	abnormal
βDDDE$_{440}$	D-D-D-E-G-E	0*	lethal
βDDDD$_{440}$	D-D-D-D-G-E	0*	lethal
βDEDD$_{440}$	D-E-D-D-G-E	0*	lethal
βDDED$_{440}$	D-D-E-D-G-E	0*	lethal
βDDED$_{442}$	E-D-D-D-G-D	18	abnormal‡
βEEEEGD$_{442}$	E-E-E-E-G-D	21	normal
βDDDDGD$_{442}$	D-D-D-D-G-D	0*	lethal
no DNA	n.a.	0*	n.a.

*In two independent experiments.
‡Phenotype appears identical to βEDDD$_{440}$.

Having determined that E$_{440}$ alone is not sufficient, we tested whether any E in the region 437–439 was sufficient for survival. We repeatedly failed to obtain transformants using fragments carrying βDEDD$_{440}$ and βDDED$_{440}$ mutations. In contrast, when cells were transformed with the βEDDD$_{440}$ mutation, we obtained high frequency rescue. However, these cells were abnormal, showing defects in shape, movement, and growth (see next section). Thus, among the four adjacent Es, only E$_{437}$ is sufficient for survival.

In *Paramecium* β-tubulin, an additional E (E$_{441}$) located downstream of a stretch of three adjacent Es, was also found to be glycylated (Vinh et al., 1999). Alignment of the COOH-terminal peptides of *Paramecium* and *Tetrahymena* β-tubulins suggests that E$_{442}$ in *Tetrahymena* β-tubulin is homologous to E$_{441}$ of *Paramecium* (Fig. 1). Therefore, E$_{442}$ also might be polyglycylated in *Tetrahymena* and *Tetrahymena* cells could require both E$_{437}$ and E$_{442}$ for survival. To test this possibility, we used a mutant gene encoding the βEDDDGD$_{442}$ mutation to transform *Tetrahymena* (Table I). Transformants carrying this mutation had an abnormal phenotype indistinguishable from that of βEDDD$_{440}$ mutants. Furthermore, mutants with a single substitution at position 442 (βEEEEGD$_{442}$) were indistinguishable from WT. Thus, E$_{437}$ is the only residue among the five COOH-terminal Es that is sufficient for survival.

βEDDD$_{440}$ Mutants Have Reduced Mono- and Polyglycylation on β-Tubulin and Increased Monoglycylation on α-Tubulin

To analyze the glycylation levels of mutant cells lacking likely polyglycylation sites, TAP 952 and AXO 49 mAbs were used to probe Western blots containing ciliary proteins. Initial analyses showed that the βDEEE$_{440}$, βDDEE$_{440}$, βEEED$_{440}$, βEEDE$_{440}$, βEDDD$_{440}$, and βEEDD$_{440}$ have polyglycylated epitopes on β-tubulin and α-tubulin (data not shown). Since the βEDDD$_{440}$ cells had a highly abnormal phenotype, the amount of glycylation in these cells was compared with that in WT cells. We first determined that mutant cilia contain similar amounts of ciliary proteins as WT cilia including α-tubulin and a membrane protein, SerH3 (Fig. 5 C). Then, Western blots containing a concentration series of WT or mutant ciliary proteins were probed with the glycylation-specific antibodies. Quantitation of Western blots showed that the amount of AXO 49 polyglycylated epitopes (three or more Gs) in the βEDDD$_{440}$ mutant β-tubulin was only 24% (±6%, n = 4) of that in WT (Fig. 5 A). Interestingly, the amount of AXO 49 epitopes were also somewhat reduced on the α-tubulin of the βEDDD$_{440}$ mutant (53.5 ± 24% of WT, n = 4). When probed with TAP 952, which recognizes primarily monoglycylated sites, no signal was detectable on the β-tubulin of βEDDD$_{440}$ mutants. In contrast, the TAP 952 epitopes increased more than twofold on α-tubulin in the βEDDD$_{440}$ mutant (Fig. 5 B).

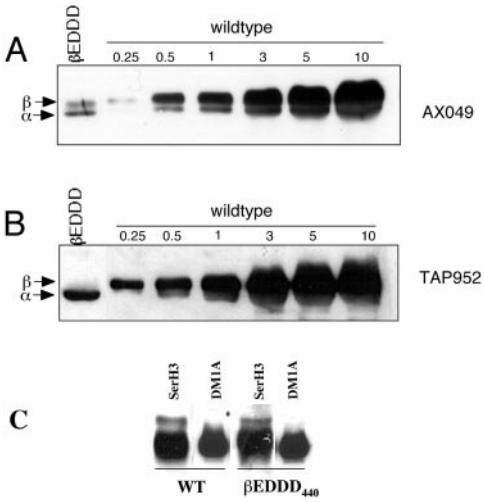

Figure 5. (A and B) Western blots of WT and $\beta EDDD_{440}$ ciliary proteins probed with antiglycylated tubulin antibodies AXO 49 and TAP952. A series of dilutions of WT ciliary proteins were run that correspond to 0.25, 0.5, 1, 3, 5, and 10× the concentration of the $\beta EDDD_{440}$ mutant ciliary proteins analyzed on the same blot. Blots were processed using the ECL system and exposed to film. A scanning densitometer to quantify the amounts of antibody reactive epitopes. The shortest exposure for which the smallest amount of WT signal could be detected was used. A standard curve was prepared using the values for the WT extracts. For the blots shown, the relative intensities of mutant signals fell within the linear range of the standard curve. (C) A Western blot of WT and $\beta EDDD_{440}$ ciliary proteins probed with polyclonal antibodies against the surface antigen of *T. thermophila*, SerH3, and monoclonal anti–α-tubulin antibodies DM1A. The same amounts of total ciliary proteins were loaded into each lane.

Growth, Motility, and Cytokinesis Are Affected in the $\beta EDDD_{440}$ Mutant

During log phase, the generation time of $\beta EDDD_{440}$ cells was ~315 min compared with 250 min in WT cells (Fig. 6 A, right). The average rate of movement of $\beta EDDD_{440}$ mutants is only 55% of WT cells, 14.2 μm/s (n = 92) compared with 25.7 μm/s (n = 94). No significant differences between length distributions of ciliary axonemes in WT and $\beta EDDD_{440}$ cells were detected (data not shown), indicating that polyglycylation did not affect cilia-based cell motility by affecting the extent of ciliary assembly.

Confocal immunofluorescence analyses of WT and $\beta EDDD_{440}$ mutant cells were performed on cells stained with antitubulin antibodies (Fig. 7). The size of $\beta EDDD_{440}$ mutant cells is more heterogeneous than WT cells (Fig. 7 A); some are smaller (Fig. 7 B) while others are bigger (Fig. 7 D). Many $\beta EDDD_{440}$ cells also have an abnormally high number of nuclei (Figs. 6 C and 7 D). In this mutant, we also observed cells in which nuclei had divided but apparently had failed to complete cytokinesis (Fig. 7 C) as well as cells with a cleavage furrow that appeared improperly positioned and shifted toward one end of the cell (Fig. 7, E and F). All of these phenotypes are consistent with defects in cytokinesis. The unusually small and large cells could be explained by unequal division, while large cells with multiple nuclei could result from cytokinesis failure.

Lethal Mutations in the Polyglycylation Region of β-Tubulin Can Be Rescued by Replacing the α-Tubulin COOH Terminus by the β-Tubulin COOH Terminus

As described above, no transformants were obtained with the $\beta DDDE_{440}$, $\beta DDDD_{440}$, $\beta DEDD_{440}$, and $\beta DDED_{440}$

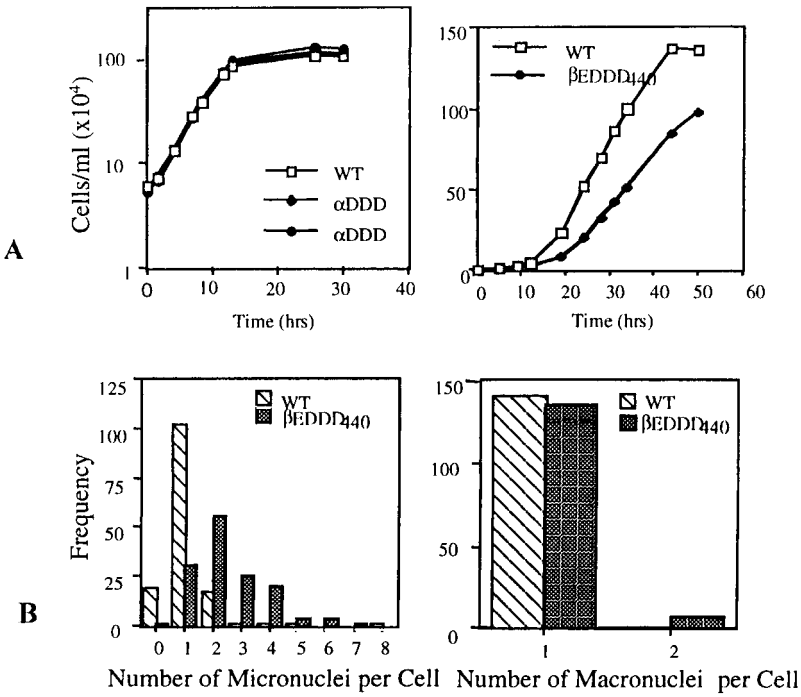

Figure 6. (A, left) Growth curves of isogenic WT and αDDD_{447} mutant cells. Cultures were grown at 30°C in SPPA medium with shaking. (A, right) Culture growth of isogenic WT and $\beta EDDD_{440}$ mutants. (B) Number of micronuclei (left) and macronuclei (right) per cell in $\beta EDDD_{440}$ mutant and isogenic WT cells.

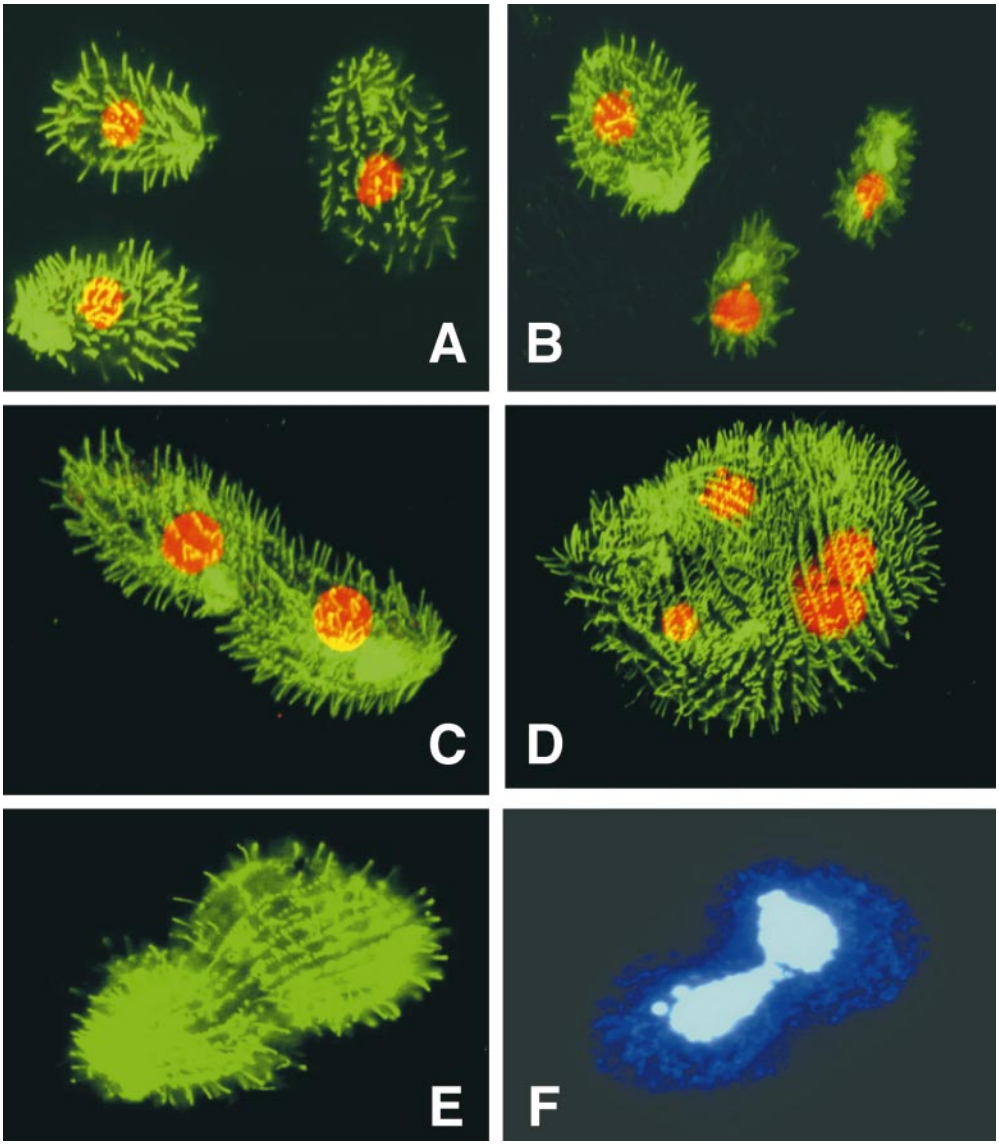

Figure 7. Confocal immunofluorescence images of wild type and $\beta EDDD_{440}$ mutants. (A–D) Cells were labeled with anti-polyG antibodies (green) and stained with propidium iodide (red). (E and F) The same cells were stained with anti-polyE antibodies (green) and DAPI (blue). (A) Wild-type cells showing normal cell shape, size, and numbers of nuclei; B–F are $\beta EDDD_{440}$ mutant cells.

mutations, suggesting that polyglycylation on β-tubulin, unlike polyglycylation on α-tubulin, is essential. Another possible explanation is that the structure of these mutant tubulins is disrupted so that they fail to assemble into microtubules. Alternatively, polyglycylation of α- and β-tubulin could have overlapping functions, and elimination of this modification from α-tubulin may be tolerated because there appears to be less polyglycylation of α- than β-tubulin (Fig. 2). If polyglycylation of α-tubulin is quantitatively insufficient to support the essential function of this PTM, it should be possible to rescue a lethal β-tubulin mutation by creating a stronger polyglycylation region on α-tubulin. However, if the defect is due to alteration in β-tubulin function unrelated to the effect of the mutation on polyglycylation, increasing the polyglycylation of α-tubulin should have no effect.

To discriminate between these alternatives, we constructed a chimeric α-tubulin gene whose COOH terminus was replaced with the corresponding region from WT β-tubulin gene (Fig. 1 C). We attempted to rescue the progeny of a mating between the double BTU knockout heterokaryons with this α-COOH terminus-β fragment alone, with $\beta DDDE_{440}$ alone, or with a mixture of the two fragments. While we did not obtain rescues with either fragment, we did obtain rescues (two transformants per 2.5 μg of each fragment) when both fragments were used simultaneously. Using PCR and DNA sequencing, we confirmed that the rescued transformants had incorporated both genes. Although the rescue frequency was low, it was in the range of frequencies of cotransformation observed previously for a drug-resistant variant of *BTU1* gene (Gaertig et al., 1994b), making it unlikely that the infrequent double transformants had acquired additional spontaneous mutations at other loci. The $\beta DDDE_{440}$ + α-COOH terminus-β cells grew more slowly than WT. But, unlike $\beta EDDD_{440}$ mutants, the $\beta DDDE_{440}$ + α-COOH terminus-β cells have normal shapes and numbers of nuclei, indicating that cytokinesis is not significantly disturbed (data not shown).

In WT cells, β-tubulin is more highly polyglycylated

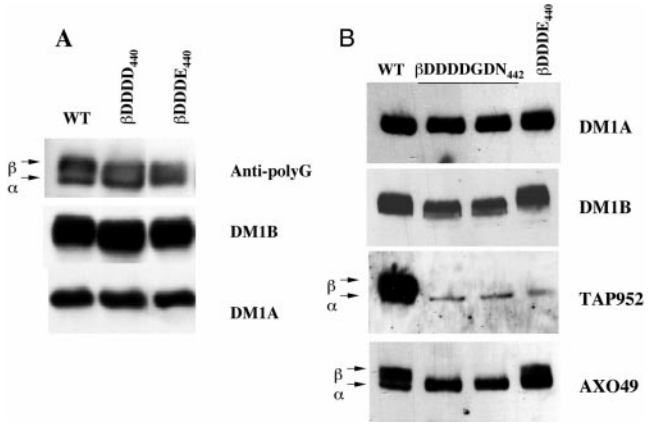

Figure 8. (A) Immunoblot of total cell proteins of WT, βDDDE440 (+ α-COOH terminus-β chimera) and $\beta DDDD_{440}$ (+ α-COOH terminus β) chimera mutants probed with the polyclonal anti-polyG antibodies (top), monoclonal anti–β-tubulin antibodies, DM1B (middle), and monoclonal anti–α-tubulin antibodies, DM1A (bottom). Approximately equal amounts of total cell proteins were loaded in each lane. (B) A Western blot of ciliary proteins of the $\beta DDDDGD_{442}$ (+ α-COOH terminus-β chimera) and $\beta DDDE_{440}$ (+ α-COOH terminus-β chimera) mutants probed with several antitubulin antibodies. Equal amounts of total proteins were loaded into each lane. The same blot was probed sequentially using the ECL system. After each exposure, the antibodies were removed, and the blot was reprobed according to manufacturer's instructions. The following antibodies were used: anti–β-tubulin, DM1B; anti–α-tubulin, DM1A; anti-monoglycylated tubulin, TAP 952; and antipolyglycylated tubulin, AXO 49.

than α. However, when ciliary proteins, which were isolated from $\beta DDDE_{440}$ + α-COOH terminus cells, were analyzed on Western blots probed with AXO 49, the amount of polyglycylation on α-tubulin was higher than that on β-tubulin, inverting the pattern seen in WT cells (Fig. 8). The fact that in the $\beta DDDE_{440}$ mutant β-tubulin is still glycylated indicates that at least one of the remaining COOH-terminal Es (E_{440} and E_{441}) is also polyglycylatable. To test whether E_{440} is a polyglycylation site, we transformed the BTU heterokaryons with the $\beta DDDD_{440}$ gene and the α-COOH terminus-β chimera. Viable double transformants were obtained, but the mutant β-tubulin was still polyglycylated (Fig. 8 A), indicating that remaining E_{442} is also a polyglycylation site. To test E_{442}, we introduced the $\beta DDDDGD_{442}$ mutation in the presence of α-COOH terminus-β chimera. Viable double mutants were obtained at a frequency comparable to the frequency of transformation for $\beta DDDE_{440}$ and $\beta DDDD_{440}$ genes. No transformants were obtained with the $\beta DDDDGD_{442}$ gene alone (Table I), and all transformants obtained in the presence of the α-COOH terminus β chimeric had incorporated both the mutant β-tubulin gene and the α-COOH terminus β chimeric gene. On a Western blot, no monoglycylation or polyglycylation was detected in the region of β-tubulin while the α-β tubulin chimera was hyperpolyglycylated (Fig. 8 B). The simplest explanation of these observations is that E_{442} was the single remaining site of polyglycylation in the $\beta DDDD_{440}$ mutant. Thus, all five COOH-terminal Es of β-tubulin ($E_{437\text{-}442}$) can be polyglycylated and the essential function of polyglycylation can be transferred from β- to the α-tubulin subunit. The phenotype of the $\beta DDDD_{440}$ and $\beta DDDDGD_{442}$ mutants was similar to the phenotype of the $\beta DDDE_{440}$ mutants (slow growth and motility but normal morphology, data not shown).

Discussion

We examined the role of the α- and β-tubulin polyglycylation sites in *Tetrahymena* using in vivo mutagenesis and immunochemical analysis with tubulin glycylation–specific antibodies. The two mAbs used, AXO 49 and TAP 952, specifically recognize glycylated tubulin polypeptides (Callen et al., 1994; Bré et al., 1996). TAP 952 has high affinity towards tubulin peptides carrying a single G attached to one or several Es of the peptide backbone, whereas AXO 49 reacts with polyglycine chains consisting of 3 or more Gs (Bré et al., 1998). Therefore, the two antibodies should detect most polyglycylated tubulins with the possible exception of those modified with 2 G residues per site. We found that α-tubulin polyglycylation detectable by these modification-specific antibodies can be eliminated completely by simultaneously replacing E_{445}, E_{446}, and E_{447} by aspartates or alanines. These observations argue that α-tubulin polyglycylation is not essential.

In the light of evolutionary conservation of tubulin glycylation in flagellated cells from evolutionarily divergent protists (Weber et al., 1996) to humans (Bré et al., 1996), it was surprising that the absence of α-tubulin polyglycylation in *Tetrahymena* had no observable phenotype. However, the polyglycylation pattern of β-tubulin appears to overlap with that of α-tubulin, suggesting that polyglycylation of α- and β-tubulin is redundant and that polyglycylated β-tubulin enabled the cells lacking α-tubulin glycylation to function normally. Our genetic analyses argue strongly that this is the case. In contrast to α-tubulin, the polyglycylation sites on β-tubulin are essential for survival in *Tetrahymena*. We showed that in *Tetrahymena*, elimination of five, four, or three glutamic acids homologous to the polyglycylation sites previously identified in *Paramecium* (Vinh et al., 1999) was either lethal or caused slow growth, slow motility, and defects in cell division, associated with considerable reduction of β-tubulin polyglycylation. The COOH-terminal Es of tubulins also can be polyglutamylated (see Introduction) in both axonemal and cytoplasmic microtubules, and this PTM was also detected in ciliates (Bré et al., 1994). Injection of an mAb specific for glutamylated tubulins led to a disassembly of centrioles in mammalian cells (Bobinnec et al., 1998). Thus, the dramatic phenotypic changes caused by substitutions of E_{437}-E_{442} in β-tubulin could conceivably result from either the reduction in the level of polyglycylation or polyglutamylation, or from a combination of both. However, in *Paramecium*, polyglutamylation must be a quantitatively minor modification since it was not detected by mass spectrometry, and only a very faint signal was detected on a Western blot with antiglutamylated tubulin antibodies (Bré et al., 1994, 1998; Redeker et al., 1994). It is likely that the same holds true for *Tetrahymena* that belong to the same class of ciliates as *Paramecium* (Baroin-Tourancheau

et al., 1992), and both ciliates have a similar design of their cytoskeletons (Fleury et al., 1992). A third possibility is that the phenotypes observed are induced by the change in the primary sequence of β-tubulin. However, this explanation also seems unlikely because all β-substitutions were charge conserving and all of the sites could be mutated singly without detectably affecting the phenotype. This argument is strengthened by the observation that lethal mutations of the polyglycylation region on β-tubulin can be expressed when the COOH terminus of α-tubulin is replaced by the COOH terminus of β-tubulin in the same cells. Therefore, the β-tubulin mutations that reduce or eliminate polyglycylation do not affect the ability of β-tubulin to form tubulin dimers or to polymerize into microtubules.

The polyglycylation-deficient mutants ($\beta EDDD_{440}$) grow slowly, have reduced motility, and frequently arrest at cytokinesis. These phenotypes could reflect either a role for polyglycylation in MT assembly or a function in already assembled microtubules. Our data argue that polyglycylation is not required for the assembly of microtubules. First, the $\beta EDDD_{440}$ mutant assembles all classes of microtubular organelles found in vegetative cells. Furthermore, cilia that have highly glycylated microtubules in WT cells, have normal lengths in $\beta EDDD_{440}$ mutant cells. A postassembly function for polyglycylation is supported by an experiment in *Drosophila* where truncation of the COOH-terminal 15 amino acids of β2 tubulin, the only isotype expressed in male germ cells, had no effect on either cytoplasmic or axonemal doublet MTs assembly in vivo (Fackenthal et al., 1993). However, the microtubules assembled from the truncated *Drosophila* β-tubulin often failed to arrange properly within the axonemes (Fackenthal et al., 1993). These data suggest that the COOH terminus of β-tubulin, and its modifications, are required for either the supramolecular organization of microtubules within organelles such as the axoneme or the function of already assembled microtubules.

The dramatic reduction in the motility in the $\beta EDDD_{440}$ mutant is consistent with involvement of polyglycylation in regulation of ciliary beating in vivo. Because the length and number of mutant cilia are normal, the simplest explanation of the observation that $\beta EDDD_{440}$ mutant cells show a dramatic reduction in the rate of cell movement is that their cilia beat more slowly. Ciliary bending is based on limited sliding of adjacent peripheral doublets caused by ATP activation of dynein arms (Satir and Barkalow, 1996). Polyglycylation could play a role in the regulation of ciliary dynein activity. Consistent with this, antipolyglycylation antibodies inhibited flagellar beating in reactivated permeabilized spermatozoa (Bré et al., 1996).

The $\beta EDDD_{440}$ mutants also show defects in cytokinesis. Thus, polyglycylation may regulate the interaction of MTs with molecular motors involved in cytokinesis. Several kinesin motors were implicated in cytokinesis (Williams et al., 1995; Ohkura et al., 1997; Raich et al., 1998). Alternatively, polyglycylation of certain MTs may be more directly involved in determining cleavage furrow positioning and/or progression without any involvement of MAPs.

An important aspect of our studies concerns the respective roles of α- and β-tubulin subunits in MTs. It is not understood why the functional subunit of MTs is a heterodimer. A heterodimer is not needed to establish polarity of a polymer since monomeric actin can form polarized filaments. Although both subunits bind a single GTP nucleotide, only GTP bound to β-tubulin undergoes hydrolysis shortly after subunit addition to an MT end, and GDP–β-tubulin plays an important role in MT disassembly (Desai and Mitchison, 1998). Studies in which MTs were decorated in vivo with purified kinesin heads showed that they bind mostly to β-tubulin with some overlap with α-tubulin (Song and Mandelkow, 1993; Hirose et al., 1996; Downing and Nogales, 1998). Thus, the β subunit appears to be more important in both regulation of MT dynamics and interaction with motors. Our results are consistent with this model. We were able to eliminate detectable polyglycylation from the α-subunit COOH terminus, but we could not obtain viable cells containing similarly mutated sites with β-tubulin. Thus, the α-subunit may be essential for the correct formation of a microtubule wall, but may play a less important role after microtubule assembly.

Our results also suggest there are functional interactions between the COOH termini of the α- and β-tubulin subunits. In the $\beta EDDD_{440}$ mutant, reduction in the extent of mono- and polyglycylation on β-tubulin was associated with a twofold increase in the extent of monoglycylation on α-tubulin. Moreover, we showed that all sites of polyglycylation on β-tubulin can be eliminated when the COOH-terminal end of α-tubulin is altered to encode the corresponding sequence of β-tubulin. Thus, an essential function of the COOH terminus of β-tubulin can be transferred onto α-tubulin. The cause of lethality of the polyglycylation region mutations on β-tubulin appears to be an insufficient total level of polyglycylation, which can be compensated if similar sites are created on α-tubulin. To our knowledge, this is the first demonstration of redundant function of the two subunits of the tubulin heterodimer.

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