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 immunological 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 glycylolation, 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 polyglycylolation, while the α-β tubulin chimera was hyperglycylated compared with α-tubulin in wild-type cells. Thus, the essential function of polyglycylolation of the COOH terminus of β-tubulin can be transferred to α-tubulin, indicating it is the total amount of polyglycylolation on both α- and β-tubulin that is essential for survival.

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

Introduction

Microtubules (MTs) 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 (Aígarana 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 polyglycylolation (Redeker et al., 1994) of α- and β-tubulin.

Polyglycylolation 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). Polyglycylolation occurs on several clustered Es of β-tubulin of Paramecium including E437, E439, E435, and E441 (Vin et al., 1999). This PTM has been found in diverse species (Rüdiger et al., 1995; Bré et al., 1996; Mary et al., 1996; Ulltigner 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 polyglycylolated tubulins inhibited the motility of reactivated sea...
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; McGath 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 μg/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 pTB5 plasmid with the coding sequence of the 3-kb HindIII fragment of BTU2 (Gaertig et al., 1993) replaced by the bacterial 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 pH A B1 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 germ line micronucleus, pTB5 or pH A B1 DNA was purified using the Plasmid Maxi kit (Qiagen). The pTB5 plasmid was linearized with EcoRI and Sal to release the btu2::bsrI insert, whereas the pH A B1 plasmid was linearized with Sac and Sal to release the btu1::neoI1 insert. Biochemical 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::bsrI transformants were selected at 60 μg/ml blasticidin S (ICN), whereas the btu1::neoI1 transformants were selected with 120 μg/ml paromomycin (pm; Sigma Chemical Co.) in SPPA. Transformants were confirmed to be heterozygous germine knockouts as described previously (Cassidy-Hanley et al., 1997). A heterozygous clone for the btu2::bsrI BTU2 was crossed to a strain heterozygous for the btu1::neoI1 BTU1 gene in the micronucleus. Double heterozygotes from this cross were mated to a B*V11 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 homzygotes 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 pTU100E3-PvuII contains the 3.2-kb HindIII genomic fragment of the α-tubulin gene of T. thermophila, ATU1 (Hai and Gorovsky, 1997). Derivatives of pTU100E3-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 in the T. thermophila gene encoding β-tubulin. The ATU1 genes (linearized with H indIII) were used to rescue mating heterokaryons strains lacking functional α-tubulin genes in the micronucleus (A A K O 2 and A A K O 5), and transformed cells were selected with 120 μg/ml pm as previously described (Hai and Gorovsky, 1997). Introduction of mutated BTU1 genes (linearized with X bal and H indIII) 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 × 10^8 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 × 10^8 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-epoxyuccynil-L-1-ε-leucylamido-(4-quinuclidinyl)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 motile, cells were centrifuged at 800 g for 3 min at 4°C. Ciliary protein concentrations were determined using the BCA assay kit (Pierce Chemical Co.).

Protein Electrophoresis and Immunoblotting

For α-tubulin mutants, axonomal 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 antibodies, we used the following mouse mAbs: TAP 952 directed against the α-tubulin, D M 1 A (1:10,000 dilution; A mershams Pharmacica); general anti-α-tubulin,
Results

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

E445 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 occurs on several clustered Es downstream of the originally identified site, E437 (Vin h et al., 1999). Two Es also immediately follow E435 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 (Hai and Gorovsky, 1997), 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).

Mutant AAA447 and DDD447 and the wild-type α-tubulin (A) and wild-type β-tubulin (B) of T. thermophila (Gaertig et al., 1993; M Cg rath 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).

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. A s a primary antibody, we used mAbs AXO 49, TAP 952, anti-polyG or anti-polyE antibodies generated against 10xE peptide at 1:100 dilution. Bodies 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 × 10^6 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 were determined using the NIH Image program.

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 αDDD447 and the αAAA447 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 E445-447 eliminated polyglycylation on α-tubulin, indicating that this modification is not essential. Mutant ATU1 genes with single and double substitutions of E445-447 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 (αAAA447 and αDDD447) 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 (αAAA447) cells. In interphase WT cells, AXO 49 mAb was 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 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 b, DM 1A (bottom).
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 biolistic 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

E437 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 E437–E442 of Tetrahymena β-tubulin (Table I). We obtained

**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).
Carrying D substitution at position 437 (b) viable, phenotypically normal progeny using fragments positions 437 and 438 (b) were obtained for the additional HindIII site (H, see diagram on the right). Consistent with gene knockout of bsvr1 in the transformed cell, a smaller 2.3-kb fragment was detected.

In the transformed cell, a smaller 2.3-kb fragment was detected that the transformant is heterozygous for the 4.8-kb fragment and the endogenous 3-kb fragment, indicating that the transformant is heterozygous for bsvr1::bsr1 (H, see diagram on the right). The transformant genomic DNA (lane 2) digested with HindIIItransformant (lane 2) probed with a 39-mer [32P]-labeled fragment containing the 3'-untranslated region of BTU2. In WT, the probe detects a 3.0-kb fragment of the gene. Gene disruption of BTU2 using the bsvr1::bsr1 fragment eliminates one HindIII site (H) and yields a fragment of 4.8 kb. (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).

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-G-E                       | 65                        | normal         |
| βDEEE440                 | D-E-E-G-E                       | 77                        | normal         |
| βEDEE440                 | E-E-E-G-E                       | 89                        | normal         |
| βEEDD440                 | E-E-D-G-E                       | 120                       | normal         |
| BDDEE440                 | D-D-E-G-E                       | 141                       | normal         |
| βEDDD440                 | E-D-D-G-E                       | 97                        | normal         |
| βEDDD440                 | E-D-D-G-E                       | 68                        | abnormal       |
| βDDDE440                 | D-D-D-G-E                       | 0*                        | lethal         |
| βDDDD440                 | D-D-D-G-E                       | 0*                        | lethal         |
| βDDDE440                 | E-D-D-G-E                       | 0*                        | lethal         |
| βDDDD440                 | E-D-D-G-E                       | 0*                        | lethal         |
| βDDDD440                 | E-D-D-G-E                       | 0*                        | lethal         |
| βDEDD440                 | E-D-D-G-E                       | 18                        | abnormal1      |
| βDEEDD440                | E-E-D-G-D                       | 21                        | normal         |
| βDEDDGD442               | E-E-G-D-G                       | 0*                        | lethal         |

*In two independent experiments.
1Phenotype appears identical to βEDDD440.

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-[32P]-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).

βEDDD440 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 βDEEE440, βDEDE440, βEDEE440, βEEDD440, and βEDDD440 have polyglycylated epitopes on β-tubulin and α-tubulin (data not shown). Since the βEDDD440 mutants 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 as WT cilia including α-tubulin were probed with the glycylation-specific antibodies. Quantitation of Western blots showed that the amount of A X 49 polyglycylated epitopes (three or more Gs) in the βEDDD440 mutant β-tubulin was only 24% (+6%, n = 4) of that in WT (Fig. 5 A). Interestingly, the amount of A X 49 epitopes were also somewhat reduced on the α-tubulin of the βEDDD440 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 βEDDD440 mutants. In contrast, the TAP 952 epitopes increased more than twofold on α-tubulin in the βEDDD440 mutant (Fig. 5 B).
Growth, Motility, and Cytokinesis Are Affected in the \(\beta E D D 440\) Mutant

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

Confocal immunofluorescence analyses of WT and \(\beta E D D 440\) mutant cells were performed on cells stained with antitubulin antibodies (Fig. 7). The size of \(\beta E D D 440\) 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 \(\beta E D D 440\) 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 Polyglycylolation Region of \(\beta\)-Tubulin Can Be Rescued by Replacing the \(\alpha\)-Tubulin COOH Terminus by the \(\beta\)-Tubulin COOH Terminus

As described above, no transformants were obtained with the \(\beta D D D 440\), \(\beta D D D 440\), \(\beta D E D D 440\), and \(\beta D D E D 440\).
mutations, suggesting that polyglycylation on β-tubulin, unlike polyglycylation on α-tubulin, is essential. A nother 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 β-DDDE440 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 β-DDDE440 + α-COOH terminus-β cells grew more slowly than WT. But, unlike β-DDDE440 mutants, the β-DDDE440 + 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 7. Confocal immunofluorescence images of wild type and βEDDD440 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 βEDDD440 mutant cells.
than α. However, when ciliarian proteins, which were isolated from βD D D E 4 4 0 + α-C O O H terminus cells, were analyzed on Western blots probed with αX O 4 9, the amount of polyglycylolation on α-tubulin was higher than that on β-tubulin, inverting the pattern seen in WT cells (Fig. 8). The fact that in the βD D D GD 4 4 2 mutant β-tubulin is still glycylated indicates that at least one of the remaining COOH-terminal Es (E E 4 4 0 and E E 4 4 1) is also polyglycylatable. To test whether E E 4 4 0 is a glycosylation site, we transformed the BTU heterokaryons with the βD D D D G 4 4 0 gene and the α-C O O H terminus-β chimera. Viable double transformants were obtained, but the mutant β-tubulin was still polyglycylated (Fig. 8 A), indicating that remaining E E 4 4 2 is also a polyglycylation site. To test E E 4 4 2, we introduced the βD D D G 4 4 2 mutation in the presence of α-C O O H terminus-β chimera. Viable double mutants were obtained at a frequency comparable to the frequency of transformation for βD D D E 4 4 0 and βD D D D G 4 4 0 genes. No transformants were obtained with the βD D D G 4 4 2 gene alone (Table I), and all transformants obtained in the presence of the α-C O O H terminus β chimera had incorporated both the mutant β-tubulin gene and the α-C O O H terminus β chimeric gene. On a Western blot, no monoglycylolation or polyglycylolation 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 E 4 4 2 was the single remaining site of polyglycylolation in the βD D D D 4 4 0 mutant. Thus, all five COOH-terminal Es of β-tubulin (E 4 3 7-4 4 2) can be polyglycylated and the essential function of polyglycylolation can be transferred from β- to the α-tubulin subunit. The phenotype of the βD D D D 4 4 0 and βD D D D G 4 4 2 mutants was similar to the phenotype of the βD D D E 4 4 0 mutants (slow growth and motility but normal morphology, data not shown).

Discussion

We examined the role of the α- and β-tubulin polyglycylolation sites in Tetrahymena using in vivo mutagenesis and immunochemical analysis with tubulin glycylation-specific antibodies. The two mAbs used, A X O 4 9 and T A P 9 5 2, specifically recognize glycylated tubulin polypeptides (C al- len et al., 1994; B r é et al., 1996). T A P 9 5 2 has high affinity towards tubulin peptides carrying a single G attached to one or several Es of the peptide backbone, whereas A X O 4 9 reacts with polyglycine chains consisting of 3 or more Gs (B r é et al., 1998). Therefore, the two antibodies should detect most polyglycylolated tubulins with the possible exception of those modified with 2 G residues per site. We found that α-tubulin polyglycylolation detectable by these modification-specific antibodies can be eliminated completely by simultaneously replacing E E 4 4 5, E E 4 4 0, and E E 4 4 1 by aspartates or alanines. These observations argue that α-tubulin polyglycylolation is not essential.

In the light of evolutionary conservation of tubulin glycylolation in flagellated cells from evolutionarily divergent protists (W eber et al., 1996) to humans (B r é et al., 1996), it was surprising that the absence of α-tubulin polyglycylolation in Tetrahymena had no observable phenotype. However, the polyglycylolation pattern of β-tubulin appears to overlap with that of α-tubulin, suggesting that polyglycylolation of α- and β-tubulin is redundant and that polyglycylolated β-tubulin enabled the cells lacking α-tubulin glycylolation to function normally. Our genetic analyses argue strongly that this is the case. In contrast to α-tubulin, the polyglycylolation 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 polyglycylolation sites previously identified in Paramecium (V inh et al., 1999) was either lethal or caused slow growth, slow motility, and defects in cell division, associated with considerable reduction of β-tubulin polyglycylolation. The COOH-terminal Es of tubulins also can be polyglutamyalted (see Introduction) in both axonemal and cytoplasmic microtubules, and this PTM was also detected in ciliates (B r é et al., 1994). Injection of an mAb specific for glutamyalted tubulins led to a disassembly of centrioles in mammalian cells (B obinnc et al., 1998). Thus, the dramatic phenotypic changes caused by substitutions of E 4 3 7-E 4 4 2 in β-tubulin could conceivably result from either the reduction in the level of polyglycylolation 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 antiglycylolated tubulin antibodies (B r é et al., 1994, 1998; R edeke et al., 1994). It is likely that the same holds true for Tetrahymena that belong to the same class of ciliates as Paramecium (B aroin-T ourancheau et al., 1994).
The polyglycylation-deficient mutants (βEDDD440) 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 βEDDD440 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 βEDDD440 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 βEDDD440 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 βEDDD440 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 reacti-

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A n 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 αα 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 αα subunit COOH terminus, but we could not obtain viable cells containing similarly mutated sites with β-tubulin. Thus, the αα 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 αα- and β-tubulin subunits. In the βEDDD440 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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