The *UNI3* Gene Is Required for Assembly of Basal Bodies of *Chlamydomonas* and Encodes δ -Tubulin, a New Member of the Tubulin Superfamily

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We have cloned the *UNI3* gene in *Chlamydomonas* and find that it encodes a new member of the tubulin superfamily. Although Uni3p shares significant sequence identity with α -, β -, and γ -tubulins, there is a region of Uni3p that has no similarity to tubulins or other known proteins. Mutant *uni3-1* cells assemble zero, one, or two flagella. Pedigree analysis suggests that flagellar number in *uni3-1* cells is a function of the age of the cell. The uniflagellate *uni3-1* cells show a positional phenotype; the basal body opposite the eyespot templates the single flagellum. A percentage of *uni3-1* cells also fail to orient the cleavage furrow properly, and basal bodies have been implicated in the placement of cleavage furrows in *Chlamydomonas*. Finally when *uni3-1* cells are observed by electron microscopy, doublet rather than triplet microtubules are observed at the proximal end of the basal bodies. We propose that the Uni3 tubulin is involved in both the function and cell cycle-dependent maturation of basal bodies/centrioles.

INTRODUCTION

Eukaryotic cells use microtubules in diverse ways: microtubules are required for chromosome segregation, for organization and movement of vesicles and organelles in the cytoplasm, and for flagellar beating. α - and β -tubulin heterodimers assemble into polymers that give rise to the microtubules of the cytoskeleton, spindle, and flagella. Assembly and establishment of microtubule patterns are under the control of the microtubule organizing center (MTOC), which is an organelle that regulates the spatial and temporal distribution of microtubules. In animal and algal cells, the MTOC consists of a pair of centrioles surrounded by pericentriolar material. Microtubules are polar, and the “plus” end, which has a faster assembly rate, is found distal to the MTOC. γ -Tubulin is the third and most recently identified member of the tubulin superfamily (Oakley and Oakley, 1989). γ -Tubulin is found at the “minus” end of the microtubules, generally localizes to the pericentriolar material, and is a part of structures that may serve as initiators for microtubule

polymerization. In *Xenopus*, these structures contain α -, β -, and γ -tubulin and at least five other polypeptides (Zheng *et al.*, 1995). γ -Tubulin has also been postulated to convert the basal body contributed by the sperm during fertilization into a centriole in *Xenopus* (Félix *et al.*, 1994; Stearns and Kirschner, 1994). The conversion of basal bodies into centrioles also occurs in each cell cycle in many green algae before mitosis (Ringo, 1967; Moestrup, 1982).

The centrioles assemble conservatively but segregate semiconservatively. At the end of cell division, each daughter cell receives a new and an old centriole (Vorobjev and Chentsov, 1982; Melkonian *et al.*, 1987; Beech *et al.*, 1988; Holmes and Dutcher, 1989). In cells injected with biotinylated tubulin, Kochanski and Borisy (1990) demonstrated that most, if not all, of the labeled tubulin was found in the new centriole. The two centrioles and the pericentriolar material of the MTOC are morphologically, immunologically, and functionally distinguishable. In many mammalian and algal cells, there are morphological differences between the structures around the two centrioles (Reider and Borisy, 1982; Vorobjev and Chentsov, 1982; Melkonian *et al.*, 1987; Beech *et al.*, 1988; Wetherbee *et*

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al., 1988; Paintrand *et al.*, 1992) that are correlated with the age of the centrioles. In mammalian cells, the pericentriolar material is concentrated around the older or parental centrioles (Reider and Borisy, 1982; Vorobjev and Chentsov, 1982). As might be expected from a functional point of view, daughter centrioles mature into parental centrioles near the beginning of mitosis and acquire pericentriolar material (Vorobjev and Chentsov, 1982). Furthermore, the grandparental centriole has additional structures and a primary cilium (Vorobjev and Chentsov, 1982). Basal bodies also show differences depending on their age. In several green algae, grandparental basal bodies nucleate elaborate and longer flagella. Parental basal bodies nucleate flagella that are longer, but not as elaborate. Daughter basal bodies nucleate short flagella (Beech *et al.*, 1987; Melkonian *et al.*, 1987). Lange and Gull (1995) identified a monoclonal antibody that distinguishes between the two centrioles in mammalian cells. An epitope is recognized on the older of the two centrioles, and the acquisition of this epitope requires one cell cycle to achieve. In *Chlamydomonas reinhardtii*, there are functional differences between the two flagella that are templated by the basal bodies. The eyespot, a structure used for phototaxis, is associated with a specialized microtubule bundle and the new basal body (Foster and Smyth, 1981; Holmes and Dutcher, 1989). In addition, the two flagella templated by the parent and daughter basal bodies respond differently to phototactic signals (Rüffer and Nultsch, 1987; Horst and Witman, 1993; King and Dutcher, 1997).

The basal bodies of *Chlamydomonas* are affected differently in strains with the *uni1* mutation (Huang *et al.*, 1982). Although the postmitotic assembly of the two flagella is normally coordinately controlled, the assembly of the two flagella becomes uncoordinated in *uni1* strains. In the *uni1* mutant strains, two basal bodies are assembled, but only one basal body assembles a flagellum (Huang *et al.*, 1982; Holmes and Dutcher, 1989). The assembly-competent basal body is always found in a specific orientation with respect to the asymmetrically assembled eyespot and is referred to as the *trans* basal body. Subsequently, it was shown that the *trans* basal body is the older or parental basal body (Holmes and Dutcher, 1989, 1992). Analysis of *uni1* strains suggested that the basal bodies play a role in providing positional information (Huang *et al.*, 1982).

We have identified two new mutations in *Chlamydomonas* that produce a uniflagellate phenotype. An increased percentage of the cells assemble a single flagellum. These mutations map to two previously undescribed loci, which are designated *UNI2* and *UNI3*. The *uni3-1* mutation was generated using insertional mutagenesis (Tam and Lefebvre, 1993) and has a molecular tag associated with the mutant phenotype. The *UNI3* gene has been cloned and encodes

the first representative of a new member of the tubulin superfamily.

MATERIALS AND METHODS

Cell Culture and Genetic Analysis

Culture conditions and media were as described previously (Lux and Dutcher, 1991); standard matings and matings with *bld2-1* cells were as described by Harris (1989) and Dutcher (1995a), respectively. Diploid strains were selected as described by King and Dutcher (1997) using the linked *nit2-1* and *ac17* mutations. The *sr1* and *pf16-2* strains were obtained from the *Chlamydomonas* Genetics Center (Duke University, Durham, NC). Transfers of cells in the pedigree experiments were performed with a braking pipette with kind instruction from Dr. David Prescott. Strains referred to as *uni1*, *uni2*, *uni3*, and *uni4* by Huang *et al.* (1982) are alleles at the *UNI1* locus and are designated as *uni1-1-uni1-4* (Dutcher, 1986).

Light and Electron Microscopy

Flagellar number counts were performed using phase optics and a 40 $\times$ objective with cells at densities of 5×10^5 to 2×10^6 cells/ml to ensure that the cells were not approaching stationary phase. Eyespots were visualized using differential interference contrast (DIC) or brightfield light microscopy (Holmes and Dutcher, 1989). The cells used for obtaining longitudinal images were processed for electron microscopy as described in Porter *et al.* (1992). Images were examined at a magnification of 21,000 $\times$ with a CM10 microscope (Philips Electronic Instruments, Mahwah, NJ) operating at 80 kV. The cells used for cross-sectional and tangential images were processed for electron microscopy as described by Goodenough and Weiss (1978).

Southern Blot Analysis and Library Screen

Chlamydomonas DNA was isolated as described by Johnson and Dutcher (1991), except that the DNA was precipitated with polyethylene glycol an additional time. Upon resuspension of the DNA pellet with Tris-EDTA, the salt concentration was adjusted to 0.8 M NaCl, and polyethylene glycol (PEG)-4000 was added to a final concentration of 7.5%. Each sample was incubated on ice for >30 min, centrifuged at 14,000 rpm for 10 min, and resuspended in water or Tris-EDTA. This extra precipitation step increased the purity of the sample and made subsequent enzyme digestions significantly more reliable. Hybridization conditions for Southern blots and library filters were described elsewhere (Johnson and Dutcher, 1991). All probes were labeled using the Multiprime DNA Labeling System (Amersham, Arlington Heights, IL).

Plasmids and Phage DNA

pARG7.8, a pBR329-based plasmid containing the *Chlamydomonas* argininosuccinate lyase gene (*ARG7*) (Debuchy *et al.*, 1989) was used in the construction of p γ Δ -1, which was used for transformation of *arg7-8* cells. pMN56, a pUC119-based plasmid containing the nitrate reductase gene (*NIT1*) was used for cotransformation/rescue experiments of *uni3-1::ARG7 NIT2 nit1-1* cells with λ -phage or genomic subclones (Fernández *et al.*, 1989; Nelson and Lefebvre, 1995). Plasmid and λ -phage DNA preparations were performed using Qiagen (Chatsworth, CA) DNA purification kits. Plasmids pCU-1 and pCU-2 were constructed in the vector pUC18. pCU-1 has a 9.5-kb insert after a digestion of λ -CU-2 with *SalI*. pCU-2 has a 5.68-kb insert made by digesting pCU-1 with *XbaI* and *EcoNI*.

Chlamydomonas Transformations

For all transformations we used the glass bead method (Kindle, 1990). Briefly, logarithmically growing 1L cultures were harvested

by centrifugation, incubated with autolysin (Harris, 1989; Dutcher, 1995b) for 20 min at room temperature to remove the cell wall, recentrifuged, and resuspended in selective medium: 525 μ l of cells, 500 μ l of acid-washed glass beads (710–1, 180 nm in diameter, Sigma Chemical, St. Louis, MO), 175 μ l of 20% PEG-4000, and 2 μ g of *Eco*RI linearized p $\Delta\gamma$ -1 DNA were vortexed at maximum speed for 30 s. Cells were allowed to recover for 2 h to overnight at 25°C and plated onto selective medium that contained ammonium nitrate and no arginine. Cotransformation experiments used 2 μ g of *Eco*RI-linearized pMN56, and 0.5, 1.0, or 2.0 μ g of each genomic λ phage. Cells were plated onto medium with sodium nitrate as the sole nitrogen source and with 0.5% top agarose to improve the transformation efficiency (Gumpel *et al.*, 1994). The transforming DNA, p $\Delta\gamma$ -1, which was used to generate the *uni3*-1 allele, contained short stretches of γ -tubulin. Although most integration events in *Chlamydomonas* are nonhomologous, it is possible that these γ -tubulin sequences may have provided homology with either α -tubulin or the *Uni3* to promote integration in this region of the genome.

Construction of the Size-fractionated Library of *uni3*-1 DNA

uni3-1 (20 μ g) DNA was digested with *Bam*HI and *Ava*I (Boehringer Mannheim, Indianapolis, IN) and fractionated on a 1% low-melting temperature agarose gel (SeaKem, Rockland, ME). Four fractions that included regions slightly above and slightly below the 2.3-kb band of the λ DNA molecular weight marker were excised and purified using β -agarase (Boehringer Mannheim). One-tenth volume of each fraction was electrophoresed on a 1% agarose gel, transferred to a Zetabind filter (Amersham), and hybridized with a single-copy γ -tubulin probe. The band from the endogenous γ -tubulin gene was significantly smaller than the aforementioned 2.3-kb band and therefore did not pose a contamination problem. The fraction with the most intense signal was ligated to the pBR329 vector and transformed into DH5 α ' electrocompetent cells. A colony containing the hybrid γ -tubulin and flanking genomic DNA sequences was isolated using standard colony hybridization techniques and the single-copy γ -tubulin sequences as the hybridization probe (Sambrook *et al.*, 1989).

Isolation of Genomic and cDNA Clones

Genomic clones were isolated from two independent libraries of *Mbo*I partially digested *arg7*-8 and wild-type DNA cloned into the *Bam*HI cloning site of λ DASHII (Stratagene, La Jolla, CA) and λ EMBL3, respectively (kind gifts of Anthony Palombella and Dr. David Johnson). Screening with a 870-bp fragment that contained flanking genomic DNA from the cloned *Bam*HI-*Ava*I DNA fragment yielded phage λ CU-1 and λ CU-2. The remainder of the walk came from screens using single-copy sequences at the ends of λ CU-2 and λ CU-3 as hybridization probes.

A cDNA library made from light-grown vegetative NO⁻ cells (a gift from Dr. Jeff P. Woessner, Washington University, St. Louis, MO) was screened with the genomic clone, and no positive clones were isolated from 500,000 plaques. cDNA libraries made from light and dark grown cells (a gift from Dr. Andy Wang, Iowa State University, Ames, IA) was screened, and no positives were isolated in 500,000 plaques. A Novagen custom cDNA library of mRNAs isolated from nitrogen-starved cells cloned into the λ EXlox vector (a gift from Dr. Rogene Schnell, University of Minnesota, St. Paul, MN) was screened with a 449-bp *UNI3* reverse transcriptase (RT)-PCR fragment (see following section). In 2.5×10^6 plaques screened, a single positive plaque was detected. The 1.75-kb cDNA clone, named Exlox53, is missing 133 bp of coding sequence and the 5' untranslated region.

Reverse Transcription PCR

Poly-A⁺ RNA was enriched from 10 μ g of total RNA isolated from wild-type cells using an mRNA isolation kit (Promega, Madison,

WI). The primers used for PCR amplification were designed from DNA sequences obtained from the rescuing genomic clone whose predicted amino acid sequence showed similarity to known tubulins using BLAST searches. The primers varied in length from 18 to 37 bases. u12 and u14 (37 and 36 bases, respectively) each contained a 9-base linker with an *Eco*RI site to facilitate cloning of PCR fragments. The sequence from the genomic clones is underlined below. These primers amplified a 449-bp fragment that was used as a probe to screen the λ EXlox cDNA library. A cDNA probe was generated because attempts to screen other libraries and Northern blots with genomic probes failed to detect a *UNI3*-specific signal. u24 and u26 (both 18 bases long) were used to extend the cDNA sequences beyond the 5' end of the 1.75-kb cDNA clone, Exlox53. The 384-bp fragment amplified by the primer combination contained 133 bp of coding sequence and 90 bp of 5' untranslated sequences not found in Exlox53. The sequence of both RT-PCR fragments was identical to the corresponding positions in the genomic clone.

All PCR reactions were performed using *Taq* DNA polymerase from Boehringer Mannheim in a Perkin Elmer-Cetus (Norwalk, CT) 480 DNA thermal reaction cyclor. mRNA (500 ng) primed with 250 ng of random hexamers (Life Technologies/Bethesda Research Laboratories, Gaithersburg, MD) was reverse transcribed with avian myeloblastosis virus-RT (Promega). One microliter of a 1:10 dilution of the first strand synthesis reaction was used in a 100 μ l reaction with 25 pmol of each primer. PCR parameters were as follows: one cycle at 97°C for 10 min and 80°C for 5–10 min before the addition of *Taq* polymerase; 10 cycles at 95°C for 1 min, 53°C for 2 min, and 72°C for 3 min; 30 cycles at 95°C for 45 s, 57°C for 1 min, and 72°C for 2 min; and one final cycle at 95°C for 45 s, 57°C for 1 min, and 72°C for 10 min. The conditions for the RT-PCR with primers u24 and u26 were similar, except that the annealing temperatures were decreased by 1°C in the first 10 cycles, and by 2°C in the middle 30 cycles and in the final cycle.

u12 (antisense): 5'CCGGAATTcagcgctcgttctccagcagcaccagc;
u14 (sense): 5'CCGGAATTcggcgctcgtgctcatcgacatggagc; u24 (antisense): 5'CCTTGTGCCAGTTGTTG; u26 (sense): 5'AG-CACGCTCTGTACTAG.

DNA Sequencing

Double-stranded DNA was made using a combined alkaline lysis-PEG precipitation method and sequenced by the DNA Sequencing Facility of Iowa State University. The cDNA clone, Exlox53, and the 384- and 449-bp RT-PCR fragments were sequenced on both strands. Sequence data were compiled using GCG Sequence Analysis Software (Madison, WI). GenBank database searches for sequence homologies were initially performed using the BLASTN program (Altschul *et al.*, 1990).

Isolation of the Genomic DNA for γ -Tubulin

The genomic DNA was isolated using degenerate PCR with primers designed to match regions of identity among the known γ -tubulins. These included *Aspergillus nidulans*, *Schizosaccharomyces pombe*, *Drosophila melanogaster*, *Xenopus laevis*, and *Homo sapiens*. Underlined sequences indicate the region that hybridize to γ -tubulin, and the remainder contain an *Eco*RI site to facilitate cloning. I indicates an inosine residue, Y indicates a pyrimidine (C or T), R indicates a purine (A or G), and N indicates any base.

g1 (sense): 5'CCGGAATTCTAYCCNGGITAYATGGAAY; g2 (antisense): 5'CCGGAATTCACYTTRTTAAIARRTG

Primers g1 and g2 generated a 773-bp PCR product that was used to screen a λ EMBL3 library (constructed by Dr. David Johnson in our laboratory) using procedures described above for the cloning of the *UNI3* gene. The clone was sequenced by the Iowa State University Sequencing Facility.

Phylogenetic Analysis of Tubulins

An alignment of 59 tubulins obtained from the SwissProt database was made by PileUp (Devereux *et al.*, 1984), CLUSTAL-W (Thomp-

son *et al.*, 1994), or Pfam (Sonnhammer *et al.*, 1997). The tree shown in Figure 7 was inferred by Fitch-Margoliash analysis using a Dayhoff PAM250 substitution matrix with PROTDIST, NEIGHBOR, and FITCH programs from the PHYLIP package, version 3.5 (Felsenstein, 1996) (J. Felsenstein [1993], PHYLIP [phylogenetic inference package], <http://evolution.genetics.washington.edu.phylip/>). Unweighted parsimony trees were inferred using the PROTPARS program from PHYLIP package (J. Felsenstein [1993], PHYLIP [phylogenetic inference package], <http://evolution.genetics.washington.edu.phylip/>). Trees generated with and without the middle sequences were qualitatively similar.

RESULTS

Phenotypic Analysis of *uni2-1* and *uni3-1*

To investigate how differences are generated between the two centrioles/basal bodies and how these differences are used to generate positional and functional differences in the cell, we screened for mutant strains that assembled only a single flagellum. Cells with a single flagellum spin in place rather than swim. This phenotype provides a rapid visual screen.

The *uni2-1* mutation was isolated after exposing cells to a brief heat shock (42°C for 40 min) and identifying strains with motility defects. The frequency of motility-defective strains was 1 in 2,000, and the *uni2-1* mutation was one of ten mutations isolated. The *uni3-1* mutation was isolated after insertional mutagenesis (Tam and Lefebvre, 1993); transformed strains were selected by virtue of arginine prototrophy conferred by integration of the *pyΔ-1* plasmid (see below; Debuchy *et al.*, 1989). A screen among about 1,000 transformants produced 20 strains with motility defects. Other strains are described elsewhere (King and Dutcher, 1997; Myster *et al.*, 1997; Ehler and Dutcher, manuscript submitted).

From light microscopic counts of flagellar number, each *Uni*[−] strain had a significant number of cells with a single flagellum (uniflagellate cells) as well as aflagellate and biflagellate cells (Table 1). We examined the *uni2-1* and *uni3-1* cells for the positioning of the flagellum; 96% and 93% of the uniflagellate cells in *uni2-1* and *uni3-1* strains, respectively, have a *trans* flagellum (n = 500). In all three known *Uni*[−] strains, the *trans* flagellum is the predominant class among the uniflagellate cells.

Genetic analysis showed that both the *uni2-1* and *uni3-1* mutations segregated 2⁺:2[−] for the flagellar phenotype, and that the two mutations were not linked to each other or to the *uni1-1* mutation (Huang *et al.*, 1982; Holmes *et al.*, 1991). In addition, arginine prototrophy conferred by the transforming DNA in the *uni3-1* strain cosegregated with the flagellar phenotype in 130 tetrads, which indicates that transforming DNA and the flagellar number phenotype are tightly linked. Both mutations were recessive to the wild-type allele and complemented mutations at the other two *UNI* loci. Each mutation appears to define a

Table 1. Flagellar number and cleavage furrow placement phenotypes of the unimutant strains

	Percentage of cells with			
	Flagella			Cleavage Defect
	Two	One	No	
Wild-type	90	2	8	1.8
<i>uni1-1</i>	5	93	2	2.0
<i>uni1-3</i>	3	90	7	1.5
<i>uni2-1</i>	23	22	55	3.2
<i>uni3-1</i>	26	25	49	15.0
<i>uni1-1 uni2-1</i>	0	0	100	2.5
<i>uni1-1 uni3-1</i>	0	0	100	35.0
<i>uni1-3 uni3-1</i>	0	0	100	39.2
<i>uni2-1 uni3-1</i>	0	0	100	23.6

Five hundred cells were counted to obtain the percentages for flagellar number and for cleavage defects. Some of the cleavage-defective cells in the single-mutant strains had more than two flagella and were not included in the cells counted for flagellar number.

newly identified locus based on mapping to known mutations. The *uni2-1* mutation maps to linkage group IX near *sr1* (112:0:0) and *pf16-2* (110:0:2). The *uni3-1* mutation maps to linkage group III between *ac17* (235:0:2) and *nit2-1* (128:0:16). One of the genes for α -tubulin (*TUA1*) also maps to this region of linkage group III (Ranum *et al.*, 1988); the *tua1-1* allele confers resistance to the antimicrotubule herbicide, oryzalin (James *et al.*, 1993). The *uni3-1* and *tua1-1* alleles are tightly linked (115:0:0) but complement each other in diploid strains. Another mutation, *supcs-1*, has a cold-sensitive lethal phenotype and maps to this region. It is tightly linked to *uni3-1* (67:0:0), and these mutations complement each other for the lethal and flagellar assembly defects. This complementation data and data below suggest that these mutations fall into different genes.

The *uni2-1* and *uni3-1* strains have phenotypes different from each other and from *uni1-1* cells when examined by electron microscopy. The basal body can be divided into two parts. It consists of the triplet microtubular cylinder of the proximal portion, and the distal portion where triplet microtubules become doublet microtubules (Ringo, 1967; Figure 1A). The transition zone between the basal body and flagellum is marked by osmophilic-staining H-shaped material in longitudinal sections of the basal bodies. In *uni1-1* strains, the daughter basal body lacks a transition zone, and the parental basal body has abnormal material present in the transition zone (Huang *et al.*, 1982). In the *uni2-1* strain, some basal bodies appeared normal. In most sections only one of the two basal bodies appeared abnormal. Based on three sections that had both basal bodies and an eyespot for orien-

Figure 1. Diagram and electron micrographs of *uni3-1* basal bodies. (A–D) Diagram of the cross-sectional images of a *Chlamydomonas* basal body based on Ringo (1967). Diagram A is the most proximal region and shows the classical cartwheel and diagram C is the most distal region of the basal body with triplet microtubules. Shown in diagram D is the appearance of doublet microtubules, which are attached to the plasma membrane. Panel E is a cross-sectional image that is likely to correspond to diagram B. Panel F is a tangential section that is represented by diagram C on the right side and diagram D on the left side. Final magnification in E and F is 125,000 $\times$.

tation, the daughter basal body was abnormal. We observed aberrant material in the transition zone as was observed in *uni1-1* sections. We also observed very short cone-shaped flagella that were never observed in wild-type or *uni1-1* strains. In the *uni3-1* strain, all basal bodies examined showed abnormalities. In wild-type cells, triplet microtubules are found near the proximal end, and the center of the basal body has a cartwheel-like structure (see Figure 1, A–D). In multiple sections from the proximal region of the basal body, triplet microtubules were not observed. Only doublet microtubules were found (Figure 1, E and F). Figure 1E is likely to be a section from a region similar to the one diagrammed in Figure 1B. Figure 1F is likely to be section that includes both regions similar to the ones diagrammed in Figure 1, C and D. Thus, the *uni3-1* mutation has an effect on the assembly of triplet microtubules in the *Chlamydomonas* basal body. The outermost or C tubule of the triplets is missing. In longitudinal section from *uni3-1* cells, we observed the accumulation of osmophilic material in the barrel of the basal body.

The *uni3-1* strain showed a cell division defect similar to the one observed in *bld2-1* cells, which fail to assemble basal bodies/centrioles. *bld2-1* cells also have defects in the coordination of the placement of the cleavage furrow and spindle. In $\sim 75\%$ of *bld2-1* cells, the spindle and cleavage furrow were mispositioned with respect to the pyrenoid, a starch-containing structure in the chloroplast, and unequally sized daughter cells were produced with misplaced eyespots (Ehler *et al.*, 1995). In *uni3-1* cells, we observed an increase in the number of cells that had misplaced eyespots in newly divided pairs of cells and unequal sizes between the pairs of sister cells. The magnitude of the cleavage defect was quantified by counting the number of cells with multiple nuclei (Table 1). Neither

uni1-1 nor *uni2-1* cells have cleavage furrow placement defects (Table 1). Thus, the Uni3 gene product is likely to be needed for assembly of triplet microtubules and is needed, directly or indirectly, for flagellar assembly and cleavage furrow placement.

To determine whether *UNI1*, *UNI2*, and *UNI3* act in the same or different pathways, we examined double mutant strains. In all combinations, we observed that the phenotypes of the double mutant strains were different than the phenotypes of the parental strains. All three double-mutant combinations were completely aflagellate (Table 1). In addition, the double-mutant combinations with *uni3-1* showed an enhanced defect in cleavage furrow placement (Table 1). It seems likely that these genes act in at least two different pathways. We also examined double-mutant strains with the *bld2-1* mutation. The *uni⁻ bld2-1* double-mutant strains all resembled the *bld2-1* single-mutant strain. The *BLD2* gene is likely to act upstream of the *UNI* genes. This result is consistent with the interpretation that the Uni gene products play a role in basal body function or assembly.

The *uni3-1* strain has a higher frequency of both aflagellate and biflagellate cells than was observed in *uni1-1* strains (Huang *et al.*, 1982 and Table 1). To address the mechanism involved in generating this pattern of flagellar number, we undertook a pedigree analysis of the flagellar phenotypes of mitotic progeny. We wanted to know whether the phenotypes occurred randomly. In other words, does the phenotype of a parental cell have an effect on the phenotypes of the progeny, or does the phenotype of the parent have no influence on the phenotypes of the progeny? As shown in Figure 2A, the phenotypes of the progeny of a particular cell are not random; the phenotype of a cell in one generation is highly predictive of the phenotypes of the two daughter cells produced during

Figure 2. Pedigree analysis of the mitotic progeny from *uni3-1* cells. Individual cells were placed in 40 μ l rich medium in microtiter wells. Upon mitotic cell division, each daughter cell was transferred into a new well, and the swimming phenotype was monitored under a dissecting microscope with 100 $\times$ magnification. (A) In the left panel, the predicted fraction of pairs of daughter cells if the phenotype is random and independent of lineage. The fractions were calculated as simple probabilities for independent events. In the right panel are the observed numbers. The fractions indicate the number of cells with the diagrammed phenotype over the number of cells successfully transferred and observed. In the three pairs that did not follow the pattern diagrammed, both cells had no flagella when observed. (B) Twelve aflagellate cells were placed individually in microtiter wells, and progeny for three successive cell divisions were monitored. The fractions indicate the number of cells that showed the diagrammed pattern over the number successfully transferred and monitored. As in part A, cells that did not show the diagrammed pattern produced two aflagellate cells.

mitosis. To confirm this result, we followed single cells through three successive cell cycles (Figure 2B). In summary, we found that aflagellate cells produced one uniflagellate and one aflagellate daughter (45 of 46). Uniflagellate (29 of 30) and biflagellate cells (23 of

25) produced one biflagellate and one aflagellate daughter cell. The phenotypes in a population of *uni3-1* cells are likely to be a reflection of the ages of the cells in the population. In the remainder of the work described, we concentrated our analysis on the molecular characterization of the *uni3-1* mutation because the allele was tagged.

Molecular Characterization of the *UNI3* Locus

In *Chlamydomonas*, transforming DNA inserts nonhomologously into the nuclear genome and provides a "tag" that can be used for isolating DNA flanking the insertion site (Tam and Lefebvre, 1993; Pazour *et al.*, 1995; Smith and Lefebvre, 1996). Many insertional events in *Chlamydomonas* are associated with the partial loss of transforming plasmid DNA as well as genome rearrangements. These genomic rearrangements include deletions as well as more complicated events (Smith and Lefebvre, 1996). The *uni3-1* mutation was generated after transformation with the plasmid p $\gamma\Delta$ -1, which carries the *ARG7* gene in a pBR329-based vector flanked by two short segments of the γ -tubulin gene (Figure 3A). These segments of the γ -tubulin gene provided insulation of the *ARG7* gene against deletion of transforming DNA and would provide single-copy probes for Southern analysis, if sequences needed for plasmid rescue were lost. In the *uni3-1* strain, a single insertion of the transforming DNA was observed using pBR329 DNA as the probe (diagrammed in Figure 3B). Southern blot analysis of the insertion was performed using pBR329 and γ -tubulin sequences as probes. The 2.5 kb at the right end of the plasmid as diagrammed contain the ampicillin resistance gene and the origin of replication; these sequences were deleted in the *uni3-1* strain. This precluded the possibility of using plasmid rescue for cloning the DNA adjacent to the site of insertion. The left end was intact and was used for constructing a size-selected library (see MATERIALS AND METHODS).

A 2.3-kb fragment with approximately 1.3 kb of flanking genomic DNA was cloned from the size-selected library made from *Bam*HI-*Ava*I-digested *uni3-1* DNA using a short γ -tubulin sequence as the probe (Figure 3C). A 870-bp *Ava*I-*Sac*I fragment derived from the flanking genomic sequence recognized a restriction fragment length polymorphism (RFLP) between the *uni3-1* strain and the parental strain. This RFLP was linked to the *uni3-1* phenotype in 16 meiotic progeny from a cross of *uni3-1* and a wild-type strain. These data support the idea that genomic DNA flanking the insertion site was cloned. This 870-bp genomic DNA was used as a probe to begin a chromosome walk in two independent genomic DNA phage libraries (see MATERIALS AND METHODS).

Four overlapping λ clones that span 54 kb of wild-type DNA were obtained from the walk that began

Figure 3. Diagram of the pΔγ-1 vector, the genomic insertion site, the size-selected genomic DNA clone with flanking sequences, and demonstration that *uni3-1* is a deletion allele. (A) The vector pΔγ-1 carries the *ARG7* gene (Debuchy *et al.*, 1989), which is diagrammed as the striped box, and pBR329 sequences, which are diagrammed as black lines. At the ends, when the plasmid is linearized with *EcoRI*, are 1049-bp and 750-bp segments of the *Chlamydomonas* γ-tubulin gene, which are diagrammed as open boxes. (B) The orientation of the plasmid at the insertion site in *uni3-1* cells. Most of the pBR329 and all of the γ-tubulin sequences were deleted from the right side as diagrammed. The 1,049 bp of γ-tubulin sequences from the left side of the pΔγ-1 plasmid remained in the transformant. (C) The cloned genomic DNA from the size-selected library made from *Aval*–*Bam*HI digested DNA from *uni3-1* cells. Probe b was used to screen the phage libraries and contains single-copy DNA from the site of the insertion. (D) Restriction map of *uni3-1* and wild-type DNA with the enzyme *NheI*. The location of probes a–f on the wild-type map are shown. Probe b in parts C and D are the same probe. (E) Southern blots of genomic DNA from *uni3-1* and wild-type cells hybridized with probes a–f. The left lane in each panel contains wild-type DNA (+) and the right lane contains *uni3-1* DNA (–).

with the 870-bp probe (labeled probe b in Figure 3, C and D). The structure of the *uni3-1* mutation was determined by hybridizing genomic DNA from wild-type and *uni3-1* cells with single-copy probes from various points along the walk (Figure 3E). The *uni3-1* hybridization patterns detected by probes a and f were indistinguishable from the wild-type patterns. For probe b, as noted above, and for probe e, RFLPs were observed. Each RFLP presumably occurred because a restriction enzyme site was missing or altered in the mutant DNA compared with the wild-type DNA

(probes b and e, Figure 3E). For probes c and d, no signal was detected in the *uni3-1* mutant DNA. Based on these data as well as Southern blots from several other probes between b and c that are not shown, we estimated that a 27-kb deletion is present in the *uni3-1* strain compared with the DNA from wild-type or parental strains.

Rescue of the *uni3-1* Strains by Transformation

To identify the *UNI3* gene, we cotransformed a *uni3-1 nit1-1 NIT2* strain with pMN56, a plasmid carrying

Figure 4. The λ phage and plasmids used for transformation rescue and in vivo deletion analysis. (A) Phage λ CU-1, λ CU-2, λ CU-3, and λ CU-4 were obtained in the chromosome walk and were used for assaying rescue after transformation. The vertical bars indicate the recognition sites for the enzyme *NheI*. The subclone pCU-1 showed rescue of the Uni^- phenotype. (B) Southern blots of phenotypically rescued *uni3-1* and control strains. Probes g, h, and i were hybridized against DNA from the rescued strains (+) and control *uni3-1* strains (-). Probes g and h are found in the same *NheI* fragment as probe d in Figure 3D. Probe i was present in all strains shown as well as 13 other rescued strains. Probes g and h were present in some, but not all, of the rescued strains. The loss of portions of the transforming DNA in rescued strains was used to further delineate the DNA needed for the restoration of the wild-type phenotype to ~ 5.5 kb.

the *NIT1* gene (Nelson and Lefebvre, 1995), and one of the four overlapping phage (λ CU-1, λ CU-2, λ CU-3, and λ CU-4) found in the walk (Figure 4A). We selected for the presence of the *NIT1* gene on medium containing sodium nitrate as the sole nitrogen source; 64, 148, 211, and 31 Nit1^+ transformants were generated in these experiments with λ CU-1, λ CU-2, λ CU-3, and λ CU-4, respectively. Because the frequency of cotransformation is often low (Ferris and Goodenough, 1994), we expected only a subset of transformants to show rescue of the Uni^- phenotype. These rescued cells would be biflagellate and able to swim; 4.7% of the cotransformants with λ CU-2 and λ CU-3 DNAs restored wild-type function, while no rescued strains were observed with λ CU-1 or λ CU-4 DNAs. Light microscopic analysis of the rescued strains revealed wild-type or nearly wild-type distributions of biflagellate, uniflagellate, and aflagellate cells and wild-type frequencies of cells with cleavage defects

($n = 500$). The presence of transforming DNA was confirmed by Southern blots of genomic DNA from 17 rescued transformants. Genomic DNA from transformants with the nonrescuing clones, λ CU-1 or λ CU-4, was also examined by Southern blots. The λ DNA was incorporated at a similar frequency, but without rescue of the mutant phenotype. Phage λ CU-2 and λ CU-3 overlapped each other by 9.5 kb, and we conclude that this overlapping region is sufficient for rescuing the Uni^- phenotype. Furthermore, this result demonstrates that the *uni3-1* allele is a null mutation as the region needed for rescue is deleted in the *uni3-1* allele.

We took advantage of in vivo deletions of transforming DNA to further define the sequences needed for rescue. Using probes g, h, and i, we found that only 5.5 kb of DNA were needed to rescue the mutant phenotypes (Figure 4B). Probes g and h were present in some, but not all, rescued transformants as shown in Figure 4B. Signal from probe i was present in all 17

Figure 5. Exon-intron distribution in the *UNI3* gene. The length of the exons (on the line) and introns (below the line) are indicated and were determined based on comparisons of cDNA and RT-PCR products with the genomic sequence. At the exon-intron boundaries, we find the sequence G GT[GA] where the boundary is indicated by a vertical line () and at intron-exon boundaries, we find the sequence CAG [GC]. Both of these sequences agree with the consensus for *Chlamydomonas* intron boundaries (LeDizet and Piperno, 1995).

strains with a rescued phenotype. Plasmid pCU-2, which has a 5.68-kb insertion, also rescued the mutant phenotype.

The *UNI3* Gene Encodes a Novel Tubulin

The 5681 bp of genomic DNA needed for rescue was sequenced, and a single-copy cDNA was isolated from a library made from nitrogen-starved cells after screening multiple cDNA libraries (see MATERIALS AND METHODS). The cDNA sequence appeared to be incomplete and was extended using RT-PCR; 133 bp of the suspected coding region and 90 bp of the noncoding 5' region were found by RT-PCR. The predicted protein has 534 amino acids with a predicted molecular weight of 55,806 and a pI of 6.6. Comparison of the 5.7 kb of genomic DNA needed for rescue and 1.97 kb of cDNA/RT-PCR sequences suggested that 10 exons and 9 introns are present (Figure 5). Each exon-intron boundary is marked by the consensus for *Chlamydomonas* introns (LeDizet and Piperno, 1995;

Figure 5). Many genes in *Chlamydomonas* show extreme codon bias (reviewed in LeDizet and Piperno, 1995). We observed a similar bias toward codons that have a G or C in the third position (88.2%).

Comparison of DNA and protein from various databases and the predicted amino acid sequence of Uni3p using several different sequence analysis algorithms suggested that this rescuing fragment encoded a tubulin (Table 2). Using the database search programs BLAST or FASTA, the first several hundred matches included only complete or partial fragments of tubulin. Using the motif search programs PROSITE or BLOCKS, the predicted amino acid sequence met the criteria for a tubulin. Using the profile method Pfam-AHMM, the predicted sequence had a high probability of being a tubulin (Table 2).

The predicted amino acid sequence of the *UNI3* gene does not correspond to the two identical α -tubulin proteins (James *et al.*, 1993), the two identical β -tubulin proteins (Youngblood *et al.*, 1984), or the γ -tu-

Table 2. Search algorithms used on Uni3p sequence

Algorithm	Result	Reference
BLAST	First 398 sequences retrieved were tubulins. Best match: sum (P) = 6.2×10^{-41}	Altschul <i>et al.</i> (1990)
FASTA	First 190 sequences retrieved were tubulins. Best match: Z score = 1.2×10^{-21}	Pearson and Lipman (1988)
BLOCKS	Match for 4/4 tubulin BLOCKS. Score = 1446 (P) < 1.3×10^{-9}	Henikoff and Henikoff (1994)
PROSITE	Match only to tubulin site [SAG]-G-G-T-G-[SA]	Bairoch (1993)
Pfam-AHMM	(P) = 2^{-140} of alignment to 198 full-length tubulin sequences by chance	Sonnhammer <i>et al.</i> (1997)

Uni3p	1	MP--CITLQL	QCQGNQLGCS	LFNTLATEFS	SHDYC---TD	AVH-----	50
Alpha-tubulin		M-REVSIHI	A.I.V.NA	CWELYCLHG	IQPDQMPS	KTIGGGDDAF	
Beta-tubulin		M-REIVHI	I.AK	FWEVVDHG	IDPTTYHG	SDL--QLERI	
Gamma-tubulin		MPREI.NL.V	V.SE	FWRKLCQHG	IAKDRLDEF	ATLGG--DRK	
Uni3p	51	EYFRPSADPN	LYTARSVLID	MEPKVVAGAR	SAAAASGSWW	RYPSSGYLVM	100
Alpha-tubulin		NTFSETGAG	KHVP.CIFL	L.T.DEV	TGTYRQ----	LFHPEQLISG	
Beta-tubulin		NVYFNE.TGG	R.VP.AI.M.	L.GTMDSV	GPYQG----	IFRPDNFVFG	
Gamma-tubulin		DV.FYO.DDE	Q.IP.AI.L	L.R.INGIQ	TSDLRN----	LENPENIFIS	
Uni3p	101	QS--GSGNNW	AQGFHGYGPO	VHEDALDLVR	KEVEHADSLT	GFLLLQSMAG	150
Alpha-tubulin		KE--DAA.F	R.HYTI.KE	IVDLA.RIR	LADNCTG.Q	LVFNAVIG.	
Beta-tubulin		QT--.A....	K.HYTE.AE	LIDSV.V.R	EA.SC.C.Q	QVCH.LG	
Gamma-tubulin		KEGG.A....	S.-YTO.EA	Q.TL.MID	BEA.YC.S.E	NMCH.IA	
Uni3p	151	GTGAGLGTYV	AEALRDEYHS	AFVANCCVWP	Y--ESGEVIV	QPYNTLLTSL	200
Alpha-tubulin		S.S.SLL	LERLSVD.GK	KSKLGFT.Y	S-POVSTAV.	E...SV.STH	
Beta-tubulin		S.M.LL	ISKIREE.PD	RMMLTFS.V	S-PKVSDTV.	E...AT.SVH	
Gamma-tubulin		S.M.SYM	LELISDR.SK	KLIQTYS.F	NQESSSDVV.	SLVTLK	
Uni3p	201	HLADVSDGLV	LENEALHRT	AAKLYGIARP	SFGDMNGIAA	RALAAALLPS	250
Alpha-tubulin		S.LEHT.VA	M.D...YDI	CRRSLDIER	TY-----	-----	
Beta-tubulin		Q.VENA.ECM	V.D...YDI	CFRTLKLT	TF-----	-----	
Gamma-tubulin		R.SLHA.AV	V.D.T.DKI	AVERLHLHK	DV-----	-----	
Uni3p	251	QPRGPYAGGA	YVAPASQQHH	HHHQAHGHGA	GPGRSSPGSS	GGGVQLPALR	300
Alpha-tubulin		-----	-----	-----	-----	-----	
Beta-tubulin		-----	-----	-----	-----	-----	
Gamma-tubulin		-----	-----	-----	-----	-----	
Uni3-tubulin	301	RADSSGGRLG	GESPGLGSLG	SRGGGATPPH	AAASSPSASG	AGGSGGWGVC	350
Alpha-tubulin		-----	-----	-----	-----	-----	
Beta-tubulin		-----	-----	-----	-----	-----	
Gamma-tubulin		-----	-----	-----	-----	-----	
Uni3p	351	TAPLAELVTR	LCGHPAYRLL	TLRSVPQLPP	ANIDFTTF--	-----	400
Alpha-tubulin		-----	-----	-----	-----TN	LNRLIAQVIS	
Beta-tubulin		-----	-----	-----	-----GD	I.H..SAVM.	
Gamma-tubulin		-----	-----	-----	-----QQ	I.S..ATVMA	
Uni3p	401	-----	-----	-----	-----	-----	450
Alpha-tubulin		-SLTASLRFD	GALNVDITEF	QTNLVYPYRI	HFMLSSYAPI	ISAEKA--YH	
Beta-tubulin		-GI.CC...P	.Q..A.LRKL	AVN.I.F..L	..FMVGFT.L	TSRGSQQ--	
Gamma-tubulin		AS-.TT..YP	.YM.N.LVGL	VAS.I.T..C	..LMTGYT.L	TAENAAGQ--	
Uni3p	451	.TWPALTKRL	RQMLVTGSVL	EEGLDWSITP	QSPGAAAALG	AGLAGPTVNR	500
Alpha-tubulin		EQLSV-ABIT	NAAFEPASMM	VKCDPL----	---HGKYMAL	CLMYRGD.VP	
Beta-tubulin		YRALTVPELT	QQMWDANKMM	CAADPR----	---HGRYLTA	SALFRGRMST	
Gamma-tubulin		-VTSNIRKTT	VLDVMRRLLQ	PKNIMVSTHT	KSRDIA-NAK	YISILNIIQG	
Uni3p	501	ALASWLILRG	QGAAEA.DVG	EFAD..PALS	AAWSPEPLSV	SYSTGRFGRG	550
Alpha-tubulin		KDVNASVATI	KTKRTIQ---	-V.WC.TGF	KCGINYQPPT	VVPGVDLAKV	
Beta-tubulin		KEVDEQMLNV	QNKNSY---	-VEWI.NNV	KSSVCDIPPK	G-----L	
Gamma-tubulin		EVDPSQVHKS	LQRIRERKQA	NIEWG.ASI	QVALSKKSPY	VQTAH-----	
Uni3-tubulin	551	AMSACLLSND	RHCVGPIORM	QEHAYGMLES	RAFVHQYEKY	GLSVAEFQDC	600
Alpha-tubulin		QRAVCMIS.S	TAIGEIPF.L	DXKFDL.YAK	..V.W.VGE	GMEEG..SEA	
Beta-tubulin		KMSATFIG.S	TAIQEMFK.V	SEQFTA.FRR	K..L.W.TGE	GMDEM..TEA	
Gamma-tubulin		RVSGMLLA.H	TSVRHLFNKV	LRDYEKLMP	KQEAQAFMQA	YRDVPR.ADA	
Uni3	601	FARIEDIAQR	YARL*	-----	-----	-----	650
Alpha-tubulin		REDLAALEKD	FEEVGAESAE	GAGEGEGEEY	*	-----	
Beta-tubulin		ESNMNDLVSE	YQQYQDASAE	EEGEFEGEEE	EA*	-----	
Gamma-tubulin		AGGGTALLEE	FADAKEVVQD	LANEYACES	ADYIQRQMMMA	S*	

Figure 6. Comparison of the predicted Uni3p with α -, β -, and γ -tubulin from *Chlamydomonas*. The sequences were aligned using the Pfam method (Sonnhammer *et al.*, 1997). The γ -tubulin sequence was generated in our laboratory (see MATERIALS AND METHODS). At the amino acid level, this γ -tubulin sequence (GenBank Accession number AF013109) differs at three amino acids positions from the GenBank entry U31545 of γ -tubulin (C. D. Silflow, University of Minnesota, St. Paul). Using the numbering in U31545, the changes are V190L, S196T,

bulin from *Chlamydomonas* (Vassilev *et al.*, 1995 and Figure 6). The predicted Uni3 protein (Uni3p) is 23%, 26%, and 27% identical to *Chlamydomonas* α -, β -, and γ -tubulin, respectively. The motif [SAG]GGTG[AG], which is the PROSITE motif for tubulins, is present in the predicted Uni3p as are other conserved regions indicated by shaded boxes in Figure 6. The sequence analysis and the lack of identity with the known tubulins of *Chlamydomonas* suggests that the Uni3 protein may represent a new member of the tubulin superfamily.

We have also compared Uni3p by domains with other tubulins (Burns, 1995). We find that the first 217 amino acids of the N-terminal region, which are encoded by the first five exons, were more similar to the γ -tubulins than they were to other tubulins. However, the Uni3p sequence is lacking some of the consensus sequences considered diagnostic of a γ -tubulin. These conserved γ -tubulin motifs are DVFFYQ at position 51, M/IIDREAE/D at position 128, VVVQPYN at position 188, VVVLN at position 209, KTTVLDMRLL at position 458, and IIQGEA at position 497 (Figure 6); they are not present in Uni3p. The last 145 amino acids, which are encoded by the last three exons, were more similar to the β -tubulins than to the other tubulins. The amino acids from 391 to 450 (as numbered in Figure 6) in the *Chlamydomonas* α -, β -, and γ -tubulins are 30% identical, but there is no corresponding region in Uni3p. Comparison of *UNI3* exons 6 and 7 failed to find any significant sequence identity in SwissProt or GenBank. However, these exons showed the same codon bias and dinucleotide frequencies as observed in other exons.

To further investigate the relationship of the Uni3 protein to the three known groups of the tubulin superfamily, we constructed phylogenetic trees using tubulins from a wide range of eukaryotic organisms (Figure 7). For the tree shown, the alignment was made using Pileup (Devereux *et al.*, 1984), and the phylogeny was constructed using the Fitch-Margoliash method (1967). We observed clustering of the α -, β -, and γ -tubulins, as expected. However, the Uni3p sequence was equally distant from the other three groups of the tubulin superfamily.

Included in the phylogenetic tree analysis were sequences of *TUB4* from *Saccharomyces cerevisiae* (Sobel and Synder, 1995) and a gamma-like tubulin from *Caenorhabditis elegans* (Sulston *et al.*, 1992). These two

genes were found in genome-wide sequencing efforts. Different groups have suggested that these genes encode either γ -tubulins (Sobel and Synder, 1995) or new members of the tubulin superfamily (Burns, 1995). In all of the trees that we constructed with Uni3p, the yeast and worm sequences fell within the cluster of γ -tubulin sequences, but had longer branch lengths than other γ -tubulin sequences (Figure 7). This result suggested that these tubulins are bona fide γ -tubulins, but are more divergent. This interpretation agrees with the analysis of Keeling and Doolittle (1996).

We have also constructed trees using UGPMA, neighbor-joining, and parsimony methods with alignments from CLUSTAL-W (Thompson *et al.*, 1994) and Pfam (Sonnhammer *et al.*, 1997). All trees generated were qualitatively the same; the Uni3p branch was always separated from the other three groups although the absolute length of the branches differed with other methods (our unpublished observations). We also constructed trees with the amino acids encoded by exons 6 and 7 of Uni3 removed and a similar number of amino acids from the other tubulins removed to determine whether the trees were strongly influenced by this nontubulin-like region. The trees were qualitatively unaffected by the absence of these sequences. Thus, it appears that the *UNI3* gene encodes a new member of the tubulin superfamily.

DISCUSSION

Chlamydomonas cells are normally biflagellate, but in the presence of mutations in any of three genes (*UNI1*, *UNI2*, or *UNI3*), an increased percentage of cells assemble zero or one flagellum. The uniflagellate cells share another property. In uniflagellate cells, the single flagellum is found on the basal body opposite the eyespot, which is the older of the two basal bodies in wild-type cells (Holmes and Dutcher, 1992). These mutations allow us to distinguish between the assembly properties of the two basal bodies, which in wild-type cells appear coordinated. This flagellar assembly phenotype in combination with the disrupted morphology of the basal bodies, as observed by electron microscopy, suggest that the Uni⁺ gene products may be required for basal body assembly.

By electron microscopy, basal bodies from *uni3-1* cells have a unique phenotype. Whereas in wild-type *Chlamydomonas* cells, the proximal portion of the basal bodies consist of a blade of three microtubules (Ringo, 1967; Johnson and Porter, 1968; Goodenough and Weiss, 1978), in *uni3-1* cells there was an absence of triplet microtubules in the basal bodies. The C tubule, in particular, appears to be missing. Triplet microtubules are considered a hallmark of centrioles and basal bodies (reviewed by Stubblefield and Brinkley, 1967). However, very few images of triplet microtubules

Figure 6 (cont). and A411R. These amino acids are indicated by double underlines in this Figure at positions 197, 203, and 584. Gaps introduced by the alignment program to optimize the alignment are indicated by (-). Identity of amino acids is indicated by (.) and a stop codon is indicated by (*). There is greater similarity/identity in the amino terminus than in the carboxy terminus. The GenBank accession number for Uni3 tubulin is AF013108.

Figure 7. Phylogenetic tree of α -, β -, and γ -tubulins from different organisms compared with Uni3p using the method of Fitch-Margoliash. Only the horizontal distances are important for determining distances. In organisms with multiple tubulin genes, the following proteins were used in the tree: TBB1 and TBA5 from *Gallus gallus*; TBB2 from *X. laevis*; TBA1 from *Paracentrotus lividus*; TBB1 and TBA1 from *D. melanogaster*; TBB1, TBA1, and TBG1 from *Zea mays*; TBB1, TBA1, and TBG1 from *Arabidopsis thaliana*; TBB1 and TBA1 from *Volvox carteri*; TBB1 and TBAE from *Physarum polycephalum*; TBA2 from *Stylonychia lemnae*; TBA1 from *Anemia phyllitidis*, TBA2 from *Mus musculus*; TBA2 from *Neurospora crassa*; TBA1 from *A. nidulans*; and TBA1 from *S. cerevisiae*.

have been published (Fulton, 1971). Centrioles have been recently observed in *Drosophila melanogaster* that have primarily singlet and some doublet microtubules during the syncytial divisions (McDonald and Morphew, 1993; Callaini *et al.*, 1997), in pole cell centrosomes (McDonald, personal communication), and in isolated centrosomes from embryos (Moritz *et al.*, 1995), but triplet microtubules have been observed during oogenesis (Mahowald and Strassheim, 1970) and spermatogenesis. Clearly, not all cells require centrioles with triplet microtubules. However, it may be that triplet microtubules are required in basal bodies to nucleate flagella. It will be interesting to determine whether triplet microtubules in centrioles are needed

in other cell types in which centrioles may have a specialized function.

The ultrastructural phenotype of *uni3-1* basal bodies suggests several possible locations for Uni3p. We have recently obtained a polyclonal serum against the Uni3p and find that it localized to the basal bodies by indirect immunofluorescence (Dutcher and Preble, manuscript in preparation). Thus, Uni3p could act as a seed for the C tubule, as a protofilament in the seam of the C and B tubules, or as a major component of the C tubule. Immunoelectron microscopy will be needed to address the localization.

The *uni3-1* cells have another phenotype that suggests they have a defect in basal body function. We

had observed previously that the correct placement of the cleavage furrow and mitotic spindle was lost in *bld2-1* cells, which fail to assemble basal bodies (Ehler *et al.*, 1995). The *uni3-1* cells, but not the *uni1-1* or *uni2-1* cells, have misplaced cleavage furrows with respect to the mitotic spindles. This phenotype suggests that different aspects of basal body function may be altered in the various *uni*⁻ mutants. Recently, a tagged allele at the *UNI2* locus was obtained. The gene was cloned, but it has no similarity to other genes in the database (Wu, Tam, Lefebvre, and Silflow, personal communication).

Uni3p Defines a New Member of the Tubulin Superfamily

The *UNI3* gene encodes a protein that represents a new class of tubulins, which clearly differs from the well known α -, β -, and γ -tubulins (Figures 6 and 7). The Uni3 tubulin differs from other tubulins in several ways. First, a cell with a deletion of the *UNI3* gene is viable; this gene is not essential. Null alleles in single-copy α -, β -, or γ -tubulin genes in a variety of organisms have lethal phenotypes (reviewed by Cabral *et al.*, 1984; Huffaker *et al.*, 1987). Southern blots of genomic DNA with genomic or cDNA *UNI3* probes did not reveal any additional hybridization signals, even under conditions of reduced stringency (Preble and Dutcher, work in progress). This result suggests that there are not additional genes with similar sequence that may provide redundant function. A second difference is the presence of two additional exons in the *UNI3* gene that encode a region not present in other known tubulins. The function of this region in the protein is unknown. These two exons contain a histidine-rich region and many glycine and proline residues.

The *UNI3* gene does not appear to be unique to *Chlamydomonas* as we have found a sequence in the mouse EST database that is likely to be a homolog. Clone 371244 (Accession no. W53427) from 13.5–14.5 d mouse embryos is 62%/51% similar/identical to Uni3p over 129 amino acids. This clone is also 43% identical to human γ -tubulin, but lacks several motifs conserved in γ -tubulins. We are currently sequencing the entire EST to determine the extent of similarity.

Burns (1995) proposed that new tubulin genes found in the sequencing projects of *C. elegans* and *S. cerevisiae* are each new members of the tubulin superfamily and named them δ and ϵ , respectively. As illustrated in Figure 7, these sequences are found in the branch with other γ -tubulin sequences, but are more divergent. The phylogenetic analysis of Keeling and Logsdon (1996) suggested that these genes represent divergent members of the γ -tubulin family rather than founding members of new subfamilies. Furthermore, functional analysis suggests that the *TUB4* gene in *S. cerevisiae* is

essential and encodes γ -tubulins (Spang *et al.*, 1996). Tub4p localizes to the spindle pole body, which is the MTOC equivalent in yeast (Sobel and Snyder, 1995; Marschall *et al.*, 1996; Spang *et al.*, 1996). The yeast genome is completely sequenced, and no tubulin genes in addition to the known α - and β -tubulin genes were found (J.M. Cherry, C. Adler, C. Ball, S. Dwight, S. Chervitz, Y. Jia, G. Juvik, S. Weng, and D. Botstein [1996], *Saccharomyces* genome database, <http://genome-www.stanford.edu/Saccharomyces/>). In *C. elegans*, no other γ -tubulin gene has been identified by the sequencing project, which is about 80% completed. The *C. elegans* γ -like tubulin gene is found in the *emb-30* operon, but definitive evidence for an embryonic lethal mutation in the γ -like tubulin gene is not yet available (Tabish, Khan, and Siddiqui, personal communication). The essential nature of the γ -tubulin gene in *A. nidulans* (Oakley *et al.*, 1990), *Schizosaccharomyces pombe* (Stearns *et al.*, 1991; Horio *et al.*, 1991), or *D. melanogaster* (Sunkel *et al.*, 1995), as well as yeast, is in contrast to the nonessential nature of the *UNI3* gene. This difference underscores the conclusion that Uni3 tubulin is unique compared with other known tubulins.

Recently another gene with a tubulin motif has been described. The *Misato* gene in *D. melanogaster* encodes a protein with both tubulin-like and myosin-like motifs (Miklos *et al.*, 1997). A null mutation in *Misato* results in flies with irregular chromosome segregation. *Misato* is no more similar to Uni3p than it is to other tubulins.

Centriole/Basal Body Maturation Requires Multiple Cell Cycles

Differences between old and new centrioles and basal bodies have been documented in a variety of organisms. Centriole/basal body maturation may be involved in promoting different structures and functions for the old (parental) and new (daughter) centrioles. In pig epithelial cells, an electron-dense halo surrounds the parental centriole during metaphase, and this centriole is perpendicular to the spindle axis. In interphase, the parental centriole has appendages, but the daughter centriole does not. This maturation requires more than one cell cycle to achieve (Vorobjev and Chentsov, 1982). In PtK₂ cells, morphological differences between the two centrioles have also been observed (Reider and Borisy, 1982; Paintrand *et al.*, 1992). In many biflagellate algal cells, the two flagella differ from one another in size, structure, and function (Melkonian *et al.*, 1987), although, to date, no morphological differences in basal bodies have been noted in green algae. The progression of flagellar morphology in these algae is postulated to require the maturation of the basal bodies that template the flagella. For ex-

ample, in *Pleurochrysis carterae*, the two flagella are functionally and structurally dissimilar. This heterogeneity requires three generations to achieve. A short, nonhairy flagellum is associated with the daughter basal body, a longer, nonhairy flagellum is associated with the parental basal body, and a longer, hairy flagellum is associated with the grandparental basal body (Beech *et al.*, 1988). The pattern of staining of the monoclonal antibody against the protein cenexin also suggests that this centriolar epitope may require more than one generation to be present on the parental centriole (Lange and Gull, 1995).

Our pedigree analysis of flagellar assembly in the *uni3-1* mutant strain is consistent with the requirement for three generations for maturation events. In the absence of the Uni3 gene product, the predominant class is aflagellate cells. We propose that these aflagellate cells contain a new basal body and a basal body that has just become a parent. This pair of basal bodies, in the absence of the Uni3 gene product, cannot assemble flagella. When a parental basal body completes another cell cycle, the pair of basal bodies assembles a single flagellum on the older basal body. When the basal body has participated in at least three cell cycles, the cell assembles two flagella.

One explanation for the pattern of flagellar assembly that we observe in the pedigree studies is that another gene product promotes assembly in the basal body pairs that have a two-generation- or three-generation-old basal body. However an alternate explanation for the pedigree observations is that the Uni3p is required for the normal segregation of basal bodies. In wild-type cells, a parental and a daughter basal body always go to each pole. If two parental basal bodies go to one pole and the two new daughter basal bodies go to the other pole, this could generate the biflagellate and aflagellate class. The uniflagellate class could arise from segregation of a parental and daughter basal body from the other parental and daughter, as we observed in *uni1-1* cells (Holmes and Dutcher, 1989). This would require that two alternative segregation patterns occurred with reproducible frequencies.

Is there a reason for a cell to distinguish between grandparent, parent, and daughter centrioles? If these centrioles play roles in establishing asymmetries in the cell, then perhaps centrosomes with different levels of maturity of their centrioles could provide positional information for the cell that could be used in orienting spindles, cleavage furrows or other cellular components. For example, the PIE-1 protein is initially localized to both centrosomes, but then disappears from the centrosome that will be in the cell destined to be a somatic cell (Mello *et al.*, 1996). It is possible that that differences in the age of the centrosomes could contribute.

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