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A mutation in the α1-tubulin gene of *Chlamydomonas reinhardtii* confers resistance to anti-microtubule herbicides

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SUMMARY

A mutation in the α1-tubulin gene of *Chlamydomonas reinhardtii* was isolated by using the amiprophos-methyl-resistant mutation *apm1-18* as a background to select new mutants that showed increased resistance to the drug. The upA12 mutation caused twofold resistance to amiprophos-methyl and oryzalin, and twofold hypersensitivity to the microtubule-stabilizing drug taxol, suggesting that the mutation enhanced microtubule stability. The resistance mutation was semi-dominant and mapped to the same interval on linkage group III as the α1-tubulin gene. Two-dimensional gel immunoblots of proteins in the mutant cells revealed two electrophoretically altered α-tubulin isoforms, one of which was acetylated and incorporated into microtubules in the axoneme. The mutant isoforms co-segregated with the drug-resistance phenotypes when mutant upA12 was backcrossed to wild-type cells. Two-dimensional gel analysis of in vitro translation products showed that the non-acetylated variant α-tubulin was a primary gene product. DNA sequence analysis of the α1-tubulin genes from mutant and wild-type cells revealed a single missense mutation, which predicted a change in codon 24 from tyrosine in wild type to histidine in mutant upA12. This alteration in the predicted amino acid sequence corroborated the ~+1 basic charge shift observed for the variant α-tubulins. The mutant allele of the α1-tubulin gene was designated *tua1-1*.

Key words: *Chlamydomonas*, α-tubulin gene, microtubules, oryzalin, amiprophos-methyl

INTRODUCTION

Microtubules are major structural components of the eukaryotic cytoskeleton and are involved in a number of processes such as nuclear and cell division, organelle transport, cell motility and maintenance of cell morphology. These highly conserved filamentous structures are composed of heterodimers of α- and β-tubulin, each with a relative molecular mass of ~50-55 kDa. A variety of microtubule-associated proteins (MAPs) are believed to determine and direct the organization of microtubules in the various processes in which they participate.

Despite the high degree of conservation in predicted protein sequence among tubulins of all species examined, tubulins of animals, plants and fungi are clearly distinguishable by certain pharmacological and immunological properties (reviewed by Morejohn and Fosket, 1986) and amino acid sequence differences (reviewed by Silflow et al., 1987; Fosket, 1989). A number of drugs bind to tubulin and disrupt microtubule function, allowing the use of a genetic approach to study microtubule function. In fungi, mutations in α- and β-tubulin confer resistance or supersensitivity to benzimidazole compounds, confirming the essential role of microtubules in mitosis, meiosis and nuclear migration (Oakley and Morris, 1980, 1981; Hiraoka et al., 1984; Toda et al., 1984; reviewed by Huffaker et al., 1987). Studies of colchicine (COL)-resistant β-tubulin mutants in Chinese hamster ovary (CHO) cells (reviewed by Schibler and Cabral, 1985a) and *Chlamydomonas* (Bolduc et al., 1988; Schibler and Huang, 1991) also have contributed to the understanding of microtubule assembly and function and of the mechanism of action of antimitotic drugs.

To gain insight into the function of plant microtubules, we have used plant-specific antimicrotubule agents to select mutants with altered microtubules in the unicellular, biflagellate green alga *Chlamydomonas reinhardtii*. This organism is ideally suited to this purpose because it possesses structurally and functionally diverse arrays of microtubules; cell cycle rearrangements of these arrays have been observed using both electron and light microscopy (e.g. see Johnson and Porter, 1968; Doonan and Grief, 1987). The two anterior flagella grow from basal bodies, which are associated with four microtubule rootlets (Ringo, 1967; Weiss, 1984). The interphase cortical array of microtubules is associated with the rootlets. The rootlet complex is bisected along with the basal body apparatus at prophase and the two basal body complexes migrate to opposite sides of the nucleus where they associate with each pole of the intranuclear mitotic spindle (Gaffal, 1988). The four-membered rootlets persist during mitosis, forming an arc over

the spindle to indicate the plane of the ensuing cytokinesis (Holmes and Dutcher, 1989; Gaffal and el-Gammal, 1990). Phycoplast microtubules, which line the cleavage furrow, appear to be focussed near the rootlets (Gaffal and el-Gammal, 1990).

Chlamydomonas has been a particularly useful system for genetic and biochemical analysis of eukaryotic flagella and the basal body apparatus (reviewed by Luck, 1984; Huang, 1986; Holmes and Dutcher, 1989), and for molecular studies of the expression of tubulin and other flagellar protein genes after deflagellation (reviewed by Lefebvre and Rosenbaum, 1986). The diversity of microtubule form and function in *Chlamydomonas* is not due to heterogeneity in the tubulin components. DNA sequence analysis showed that the two β-tubulin genes code for identical proteins (Youngblom et al., 1984), whereas the products of the two α-tubulin genes were predicted to differ at only two residues (Silflow et al., 1985).

To identify genes whose products may participate in microtubule-related processes, we selected mutants resistant to the antimicrotubule herbicides amiprophos-methyl (APM) and oryzalin (ORY). Because *Chlamydomonas* is extremely sensitive to the drugs (Fedke, 1982; James et al., 1988) and because previous studies demonstrated that the primary mode of action of the drugs involves specific binding to plant tubulin and disruption of plant microtubules in vivo and in vitro (reviewed by Morejohn and Fosket, 1986; Morejohn et al., 1987), we expected that some mutants would possess alterations in the genes for α- or β-tubulin. The finding that COL-resistant β-tubulin mutants of *Chlamydomonas* also conferred resistance to APM and ORY (Bolduc et al., 1988) supported this prediction. Our initial mutagenesis experiments produced mutants with varying degrees of APM and ORY resistance, some of which expressed strong conditional phenotypes and resistance or collateral sensitivity to other antimicrotubule drugs. None of the loci that could be mutated to increase resistance to APM or ORY was linked to any of the four tubulin genes, which had been mapped previously by restriction fragment length polymorphism procedures (Ranum et al., 1988; James et al., 1988, 1989). It was possible that mutations in tubulin genes were not detected in these earlier experiments because the level of drug resistance caused by mutations in tubulin genes was insufficient for detection by the selection protocol. To obtain additional mutations, we used a step-up selection protocol in which cells containing a single resistance mutation were mutagenized and subjected to selection for higher levels of drug resistance. Similar step-up selection protocols have been used to obtain multi-drug-resistant CHO cell lines displaying altered membrane permeability and altered expression of the 170-180 kDa membrane P-glycoprotein (Kartner et al., 1983). In this study, an APM- and ORY-resistant *apm1* allele (James et al., 1988) was used as a background to select two new *Chlamydomonas* mutations that caused increased resistance to APM. One of the mutations conferred pleiotropic drug resistance, and was described elsewhere (James and Lefebvre, 1989). Reported here is the characterization of a step-up mutation, upA12, that synergistically enhances APM resistance in combination with *apm1* alleles. We demonstrate that mutant upA12-bearing strains express electrophoretically altered α-tubulins due to a single amino acid substitution in a conserved region of the α1-tubulin gene. Results obtained in this study led to a re-examination of cDNA and genomic clones encoding α-tubulin and to the discovery that the two α-tubulin genes in wild-type *Chlamydomonas* cells encode identical proteins.

MATERIALS AND METHODS

Strains, culture conditions and genetic analysis

Genotypes of *Chlamydomonas* strains used in this study are listed in Table 1. Culture conditions, media, tetrad analysis and diploid analysis were carried out as previously described (James et al., 1988, 1989; James and Lefebvre, 1992). Diploids were selected using complementing nitrate reductase-deficient mutations *nit1* and *nit4* (Fernandez and Matagne, 1986). Use of the antimicrotubule agents APM, ORY, COL and taxol (TAX) and other inhibitors was described previously (James et al., 1988, 1989; James and Lefebvre, 1989, 1992). Because of the low solubility of TAX (~28-30 μM), DMSO was added to a final concentration of 1.0-1.6% in all TAX-containing media. Cell growth and viability on agar media were unaffected by DMSO at concentrations below 2.0%. To test for possible non-specific, pleiotropic drug resistance, mutant upA12 was tested for resistance or sensitivity to the four antimicrotubule agents, as well as to the cytoplasmic protein synthesis inhibitors cycloheximide, anisomycin and paromomycin; the organellar protein synthesis inhibitors erythromycin and streptomycin; the photosynthesis inhibitor atrazine; and the nonionic detergent Nonidet P-40. For each drug, the mutants were tested over a range of concentrations, which included levels below, at and above the threshold lethal dose for wild type. The mini-

Table 1. *Chlamydomonas* strains

Strain	Genotype	Source
NO^+ (wild-type)	*mt*$^+$ *nit1nit2*	U. Goodenough
$NO5c^+$ (wild-type)	*mt*$^+$ *nit1nit2*	P. Lefebvre
$NO5d^-$ (wild-type)	*mt*$^-$ *nit1nit2*	P. Lefebvre
$21gr^+$ (wild-type)	*mt*$^+$ *nit*$^+$	J. Rosenbaum
EF104 (wild-type)	*mt*$^+$ *nit4*	E. Fernandez
CC-1387	*mt*$^+$ *pf27nit1nit2*	The Chlamydomonas Genetics Center
CC-1116	*mt*$^+$ *pf5 nit2*	The Chlamydomonas Genetics Center
SJ56-A2	*mt*$^+$ *apm1-1 nit1nit2*	James et al. (1988)
SJ652-A12	*mt*$^+$ *apm1-7nit1nit2*	James et al. (1988)
SJ656-A9	*mt*$^+$ *apm1-18nit1 nit2*	James et al. (1988)
SJ531-A12	*mt*$^+$ *apm2-1nit1nit2*	James et al. (1988)
MT185-4A	*mt*$^+$ *ory1-2 nit4*	James et al. (1989)
SJ873-B1	*mt*$^-$ *pdr1-1* (up6.5) nit$^-$	James and Lefebvre (1989)
SJ871-A11	*mt*$^+$ *pdr2-1 nit4*	James and Lefebvre (1989)
SJ-C6*	*mt*$^+$ *ac17 sr1* nit$^+$	This study
SJ863-D6	*mt*$^-$ upA12 *nit2*	This study
SJ852-C1	*mt*$^+$ upA12 *pyr1sr1* nit$^-$	This study
SJ852-A4	*mt*$^-$ upA12 nit$^-$	This study
SJ861-C2	*mt*$^-$ upA12 nit$^+$	This study
SJ879-A10	*mt*$^+$ upA12 nit$^+$	This study
SJ887-A11	*mt*$^+$ upA12 *nit4*	This study
SJ934-C2†	*mt*? upA12 *nit2*	This study
SJ934-F7†	*mt*? upA12 *nit2*	This study

*The *ac17* and *sr1* markers in this strain originated from Chlamydomonas Genetics Center strain CC-29 (*mt*$^-$ *ac17can1 nic13 pf2 y1 pyr1msr1act2 sr1nit1nit2*).

†These strains were progeny derived from crosses of *C.reinhardtii ac17* upA12 *nit2* (SJ878-C8)by the polymorphic wild isolate S1-D2 (Gross et al., 1988).

mum lethal dose, or threshold lethal dose, was estimated as the minimum dose required to kill 100% of cells grown on minimal agar medium.

Mutagenesis and mutant isolation

The recessive APM-resistant mutant *apm1-18* (James et al., 1988) was used as a background for selecting strains that exhibited increased resistance to APM after mutagenesis. The step-up selection procedure was described previously (James and Lefebvre, 1989), as was the ultraviolet (UV) light mutagenesis protocol (James et al., 1989).

Flagellar regeneration

These experiments were performed using methods described by Lefebvre et al. (1978). To test the effects of APM on regeneration, the drugs were added to cultures within one minute after deflagellation. To prevent loss of APM by binding to glass surfaces, all culture vessels were siliconized prior to use.

Preparation of cell extracts and two-dimensional gel electrophoresis

Protein extracts were prepared as follows: 2×10^7 cells were pelleted, placed on ice, and lysed by the addition of 3.6 ml of cold (4-8°C) 2× sodium dodecyl sulfate (SDS) sample buffer containing 125 mM Tris-HCl, pH 6.8, 20% (w/v) glycerol, 10% (v/v) β-mercaptoethanol, 4% (w/v) SDS (FSA Laboratory Supplies, Loughborough, England), followed by vortexing vigorously for 2.5 min. The samples were centrifuged for 15 min at 17,000 ***g*** at 4°C to pellet cell debris. Supernatant proteins were precipitated by the addition of 36 ml acetone at −20°C. Precipitates were centrifuged at 0°C for 8-10 min at 3200 ***g***. The pellets were washed twice with cold acetone, and then air-dried. Protein pellets were suspended by addition of 50 µl lysis buffer (5 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.6, 1% (w/v) Nonidet P-40), 5 µl of 2 mg/ml leupeptin (Sigma, St. Louis, MO), and 3 µl of 2.5 mg/ml DNase (Worthington, Freehold, NJ). After incubation on ice for 15 min, a 50 µl sample of the protein slurry was combined with 63 mg urea and 100 µl of Solution A (2% (w/v) Nonidet P-40, 5% (v/v) β-mercaptoethanol, 9.5 M urea, and 2% (v/v) Ampholines (Serva, Paramus, NJ) in a 2:3 ratio of pH ranges 3-10 and 5-6). The samples were solubilized by vortexing at room temperature, then clarified by centrifugation in a microcentrifuge. Supernatants were transferred to new tubes, and were immediately frozen at −80°C. This method was adapted from Hussey et al. (1988). Five to 15 µl of the sample preparations was loaded onto first-dimension gels.

Flagellar axonemes were isolated from cells after dibucaine-induced flagellar amputation, as described by Witman et al. (1982). Axonemal proteins were solubilized as described above by adding 250 µl lysis buffer, 320 mg urea and 500 µl Solution A, and then stored at −80°C.

Two-dimensional polyacrylamide gel electrophoresis was performed using the method of O'Farrell (1975), with modifications described by Burland et al. (1983). First-dimension isoelectric focusing (IEF) tube gels (14.0 cm × 2.5 mm) were composed of 0.55 g/ml urea, 2% NP-40, 13.3% acrylamide (30% (w/v) stock, containing 28.4% acrylamide, 1.62% bisacrylamide), and 5% (v/v) Serva Ampholines in a 2:3 ratio of pH 3-10 and 5-6. First-dimension gels were run at 400 V for 16.5 h, then 800 V for 3 h. Prior to loading second-dimension SDS slab gels (Laemmli, 1970), the tube gels were equilibrated at room temperature for 40-45 min in SDS sample buffer. Second dimension gels (~17 cm wide × 14.5 cm high × 1.5 mm deep) were formed with a 13 cm high-resolving gel containing 10% acrylamide and a 1.5 cm high-stacking gel containing 4.5% acrylamide. The gels were run for 17 h at 10 mA.

Immunoblotting

Proteins were transferred from second-dimension slab gels to nitrocellulose using a model TE 50 Transphor apparatus (Hoefer Scientific Instruments, San Francisco, CA) in the buffer described by Towbin et al. (1979). Transfer was carried out for 16-24 h at 0.1 amps (~25 V). Immunostaining was performed as described by Birkett et al. (1985), using three mouse monoclonal antibodies generously provided by G. Piperno (Mount Sinai School of Medicine, New York). The antibody 6-11B-1 recognizes the acetylated form of α-tubulin (Piperno and Fuller, 1985); the B-5-1-2 antibody recognizes both non-acetylated and acetylated α-tubulins (LeDizet and Piperno, 1987); and 2-10-B6 recognizes β-tubulins (Joyce et al., 1992). A goat anti-mouse IgG conjugated with horseradish peroxidase (Bio-Rad, Richmond, CA) was used to visualize the staining pattern of the anti-tubulin antibodies.

RNA isolation and cell-free translation

Total *Chlamydomonas* RNA was isolated from cells that were deflagellated mechanically and then allowed to regenerate for 60 min in order to induce maximal accumulation of mRNA for tubulins. RNA was isolated as described by Schloss et al. (1984). In vitro translation of total RNA using the rabbit reticulocyte lysate system and [^{35}S]methionine was performed as previously described (Lefebvre et al., 1980). Translation mixtures were precipitated in 9 volumes of −20°C acetone and the precipitates were resuspended in 10 µl lysis buffer (described above), 2 µl of 2 mg/ml leupeptin, 10 µl of 2.5 mg/ml DNase, and 1 µl of 1 mg/ml RNase A (Worthington). After 15 min of incubation at room temperature, 10 mg urea and 20 µl of Solution A (described above) were added, protein and urea were solubilized by vortexing, and the mixtures were stored at −80°C. IEF gels were loaded with 20-30 µl of sample containing 2000-4000 cpm/µl. Two-dimensional gel protein profiles were clearly visible after autoradiography of the dried gels for 24-48 h using Kodak X-Omat AR X-ray film.

Isolation of α1 and α2-tubulin genes from mutant and wild-type cells

The two α-tubulin genes from mutant upA12 and from wild type (strain NO5c$^-$) were obtained from partial genomic DNA libraries. The α1-tubulin gene resides on a ~5 kb *Eco*RI - *Xho*I DNA fragment (Silflow et al., 1985). The α2-tubulin gene resides on two *Pst*I fragments of approximately 4.0 kb and 1.0 kb (Ranum et al., 1988). To construct partial libraries containing these fragments, DNA from wild type and from mutant upA12 cells was digested to completion with the appropriate enzymes and electrophoresed on low melting point agarose gels. DNA fragments in the desired size range were excised and ligated with pUC118 or pUC119 plasmid DNA digested with the appropriate enzymes (Viera and Messing, 1987). The ligation mixtures were transformed into *Escherichia coli* strain MC1061 and colonies were screened for the presence of *Chlamydomonas* α-tubulin sequences using colony hybridization (Gergen et al., 1979).

DNA sequencing

Recombinant plasmids were transformed into the F′ host strain MV1190 and single-stranded template DNA was prepared as previously described (McMullen et al., 1986). DNA sequencing reactions were carried out using Sequenase (U.S. Biochemical Corp., Cleveland, OH) following the manufacturer's instructions. In some reactions, double-stranded DNA from CsCl gradient-purified plasmids was used as a sequencing template. Sequencing reactions were primed with the M13 universal primer or with one of seven different synthetic oligonucleotide 17mers, which together allowed the entire sequence of both α-tubulin genes to be obtained.

RESULTS

Isolation and characterization of an APM^r ORY^r step-up mutation

We originally isolated many alleles of *apm1* and two alleles of *apm2* by directly selecting for mutants resistant to the herbicide APM (James et al., 1988). To identify other genetic loci that could be mutated to confer APM resistance, we carried out a pilot experiment in which the *apm1-18* mutant was screened for stepped-up resistance to APM after UV mutagenesis. The threshold lethal dose of APM for cells carrying the *apm1-18* mutation was 3.0 μM, approximately six times the lethal dose for wild-type cells. By selecting on media containing 6.5 μM APM, a double mutant strain up6.5-*apm1-18* was isolated; the pleiotropic drug resistance phenotype of the up6.5 mutation was described earlier (James and Lefebvre, 1989). By selecting for growth of the mutagenized *apm1-18* cells on 8.5 μM APM, we recovered a second strain, upA12 *apm1-18*, that exhibited stepped-up APM resistance and is described below.

Tetrad analysis of outcrosses of upA12 *apm1-18* revealed the segregation of a new Mendelian APM^r mutation, upA12, unlinked to *apm1-18*. Four phenotypic classes were observed among the outcross progeny of 60 tetrads in a roughly 1:1:1:1 ratio, as follows: (1) inferred wild-type (+ +) cells that were drug-sensitive, dying at 0.5 μM APM; (2) inferred *apm1-18* single mutants, exhibiting 5- to 6-fold APM resistance (2.5-3.0 μM APM); (3) inferred upA12 single mutants, displaying relatively weak twofold APM resistance (1.0-1.2 μM APM); and (4) inferred *apm1-18* upA12 double mutants that grew well at a high APM concentration (6.0 μM).

We examined the growth of upA12 cells on a variety of drugs that affect microtubule function. upA12 single mutant progeny exhibited twofold resistance to APM and ORY, and showed twofold greater sensitivity to TAX than wild-type strains, dying at 10-14 μM (Table 2). upA12 single mutants displayed wild-type sensitivity to colchicine (data not shown). Progeny bearing the single APM^r ORY^r TAX^{sst} mutation upA12 were crossed to wild-type strains, and tetrads were analyzed for the segregation of the multiple drug phenotypes. In all tetrads, the mutant phenotypes segregated 2:2 and the three drug-related phenotypes co-segregated (ORY^r vs TAX^{sst}, n=60; APM^r vs ORY^r, n=276), indicating that they resulted from a single nuclear lesion. The mutants displayed the same sensitivity as wild-type cells to seven inhibitors (see Materials and Methods) whose actions are unrelated to microtubule function (data not shown). Thus upA12 does not confer a pleiotropic drug-resistance phenotype as seen, for example, for the *pdr1-1* mutation (James and Lefebvre, 1989).

Table 2. The upA12 mutation confers resistance to APM and ORY and hypersensitivity to TAX

	Threshold lethal dose (μM)		
Strain*	APM†	ORY‡	TAX§
NO5c$^-$ (WT)	0.5	5.0	22.0
apm1-18	3.0	25.0	22.0
upA12	1.0	9.5	12.0
apm1-18 upA12	10.0	40.0+	16.0

*At least four different strains of each mutant or wild type were tested for resistance or supersensitivity to the antimicrotubule drugs.

†Resistance was quantified by plating cells on plates containing APM in 0.3 and 0.5 μM increments, over the range 0.3-10.0 μM.

‡Resistance was quantified by plating cells on plates containing ORY in 0.5 and 1.0 μM increments, over the range 4.0-10.0 μM; and in 2.5 μM increments, over the range 10.0-40.0 μM.

§Resistance was quantified by plating cells on plates containing TAX in 2.0 and 3.0 μM increments, over the range 3.0-25.0 μM.

Resistance to APM was increased in a synergistic manner in strains containing both the upA12 and *apm1-18* mutations. The highest level of APM resistance for double mutants was 9-10 μM, or approximately 20-fold greater than wild-type cells; this resistance is 2.5 times higher than the predicted resistance levels if the effect of the two mutations was additive (Table 2). The highest level of ORY resistance could not be determined due to its low solubility, but double mutants grew vigorously on 40 μM ORY. Double mutants were nearly as sensitive to TAX as upA12 single mutants. The upA12 mutation increased resistance in a synergistic manner with two other *apm1* alleles, *apm1-1* and *apm1-7* (Table 3), indicating that the upA12-*apm1-18* interaction is not allele-specific. A synergistic increase in drug resistance was also observed in strains carrying the upA12 and *pdr1-1* mutations. In contrast, double mutants obtained from crosses of upA12 by *apm2-1* (James et al., 1988), *ory1-2* (James et al., 1989) and *pdr2-1* (James and Lefebvre, 1989) exhibited additive, or nearly additive, increases in APM resistance (Table 3). The upA12 mutation was semi-dominant for the three drug phenotypes (Table 4). Diploids heterozygous for the upA12 mutation (upA12/+) expressed levels of APM and ORY resistance intermediate between homozygous wild type and homozygous mutant (upA12/upA12) diploids, and heterozygous (upA12/+) diploids displayed hypersensitivity to TAX.

The upA12 mutation was mapped to linkage group III using two- and three-factor crosses (Table 5). upA12 was unambiguously located in the ~4 cM centromere-proximal interval between *ac17* (acetate requiring) and *pf5* (paralyzed flagella), the same region to which the α1-tubulin gene had been mapped previously (Ranum et al., 1988). For this reason, the α-tubulins in upA12-bearing strains were analyzed by two-dimensional gel electrophoresis to detect possible electrophoretic variants.

Mutant upA12 expresses electrophoretically altered α-tubulin

The α-tubulin proteins in whole cell protein extracts of wild type and mutant upA12 were compared by probing immunoblots of two-dimensional polyacrylamide gels with a monoclonal antibody directed against α-tubulins. Previous studies have shown that wild-type *Chlamydomonas* cells contain two α-tubulin isotypes, termed α1 and α3, and a single β-tubulin isotype (Lefebvre et al., 1980; Silflow et al., 1982). The α3 isotype is generated by post-translational acetylation of the primary α-tubulin gene product, the α1 isoform (Lefebvre et al., 1980; L'Hernault and Rosenbaum, 1985). The identical α1 isoform is encoded by the two α-tubulin genes in *Chlamydomonas* (see below). Using an antiserum that recognizes α-tubulins (Ledizet and Piperno,

Table 3. Phenotypes of double mutants containing upA12 and other APM-resistance mutations

Cross	Threshold lethal dose of APM (μM)*						
	upA12	*apm1*	*apm2*	*ory1*	*pdr1*	*pdr2*	Double-mutant†
upA12 × *apm1-1*	~1.0	~3.0	–	–	–	–	8.0-10.0
upA12 × *apm1-7*	~1.0	~3.0	–	–	–	–	8.0-10.0
upA12 × *apm1-18*	~1.0	~3.0	–	–	–	–	8.0-10.0
upA12 × *apm2-1*	~1.0	–	1.5-2.0	–	–	–	2.0-3.0
upA12 × *ory1-2*‡	~1.0	–	–	0.5-0.6	–	–	1.5-2.5
upA12 × *pdr1-1*	~1.0	–	–	–	2.5-3.5	–	7.0-10.0
upA12 × *pdr2-1*	~1.0	–	–	–	–	5.0-7.0	7.0-10.0

*Resistance was quantified by plating tetrad progeny on agar media containing APM at the following concentrations: 0.5, 0.6, 0.8, 1.0, 1.2, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 and 12.0 μM.

†Double mutant progeny were identified as the resistant members of NPD type tetrads or by the segregation of progeny in NPD and T type tetrads.

‡Because *ory1-2* is nearly sensitive to APM, the ts-lethal phenotype of this mutant was used for scoring *ory1* segregation in crosses to mutant upA12.

Table 4. The upA12 mutation is semi-dominant

Diploid strain*	Threshold lethal dose (μM)			Interpretation
	TAX†	APM‡	ORY§	
+/+	18.0	0.3	4.0	–
apm1-18/+	18.0	0.3	4.0	Recessive
apm1-18 upA12/+ +	18.0	1.0	7.5	Semi-dominant
upA12/+	10.0	0.7	7.0	Semi-dominant
upA12/upA12	12.0	1.0	10.0	–

*15 diploid clones from each diploid construction were recovered and analyzed.

†Resistance was quantified by plating cells on plates containing 10.0, 12.0, 15.0, 18.0, 21.0, 24.0 and 27.0 μM TAX.

‡Resistance was quantified by plating cells on plates containing APM in 0.1 μM increments, over the range 0.2-1.2 μM.

§Resistance was quantified by plating cells on plates containing ORY in 0.5 and 1.0 μM increments, over the range 3.0-9.0 μM.

Table 5. The upA12 mutation maps to the same interval as the α1-tubulin gene on the left arm of linkage group III

Cross	PD:NPD:T	Distance (cM)
Centromere distance		
upA12 × *ac17**	92:0:3	1.6
upA12 × *pf27**	55:70:15	5.4
Mapping on linkage group III†:		
upA12 × *ac17*‡	92:0:3	1.6
upA12 × *nit2*§	251:0:33	5.8
ac17 × *nit2*¶	76:0:19	10.0
upA12 × *pf5*‖	252:0:15	2.8
nit2 × *pf5*‖	183:0:6	1.6

**ac17* is tightly linked to the centromere of linkage group III (< 1.0 cM); *pf27* is tightly linked to the centromere of linkage group XII (< 2.0 cM). These markers were used to estimate the centromere distance of mutant upA12, using the formula 1/2 T/total tetrads (Gowans, 1965).

†Locus-to-locus distances were calculated using the formula 1/2 T + 3 NPD/total tetrads (Gowans, 1965).

‡Data compiled from two- and three-factor crosses (upA12 *nit2* × *ac17 sr1* [nit$^+$]).

§Data compiled from two three-factor crosses (upA12 *nit2* × *ac17sr1* [nit$^+$]; and upA12 [nit$^+$] × *pf5nit2*).

¶Data compiled from one three-factor cross (upA12 *nit2* × *ac17sr1* [nit$^+$]).

‖Data compiled from two- and three-factor crosses (upA12 [nit$^+$] × *pf5 nit2*).

1987), wild-type cells were shown to express two α-tubulin isoforms, as anticipated (Fig. 1A), but cells of mutant upA12 expressed four α-tubulin isoforms (Fig. 1B). This pattern was observed consistently in protein preparations made from cells of a number of different strains containing the upA12 mutation. Immunoblots were also probed with a β-tubulin antiserum; mutant and wild-type cells possessed one β-tubulin isoform, which resolved as a single discrete spot (data not shown). The distance between β-tubulin and the α3 wild-type isoform and the distance between β-tubulin and the most acidic isoform of mutant upA12 (putative α3) were identical, as judged from overlays of gel negatives prepared from wild-type and mutant proteins. These observations suggested that two of the four α-tubulin species in mutant upA12 were the same as the α-tubulin isoforms seen in wild-type cells. To test this possibility, mutant and wild-type protein preparations were combined in the same gels in various ratios. The wild-type α1 and α3 isoforms co-migrated with the putative wild-type isoforms of mutant upA12 (Fig. 1C and D), demonstrating that mutant upA12 expresses both wild-type α-tubulin (α1 and α3) isoforms as well as two mutant isoforms, designated α1m and α3m. The mutant products show an approximate +1 (basic) charge shift relative to the wild-type products.

We used a monoclonal antibody specific for acetylated α-tubulin (6-11B-1; Piperno and Fuller, 1985) to test the possibility that the α3m isoform was produced by acetylation of the α1m isoform. In proteins from wild-type (NO5c$^+$) cells, the 6-11B-1 antibody recognized only the α3 isoform (Fig. 1E). In proteins from upA12 mutant cells, the 6-11B-1 antibody recognized both the α3 and α3m isoforms (Fig. 1F). Because the precursor-product relationship between the α1 and α3 isoforms had been established previously, these results suggested that α3m is an acetylated form of α1m.

To establish that the α-tubulin protein polymorphisms that we observed were genetically linked to the herbicide-resistance phenotype of the upA12 mutant, it was crossed to wild-type. The progeny from each of four tetrads were recovered and analyzed by 2-D immunoblotting of whole

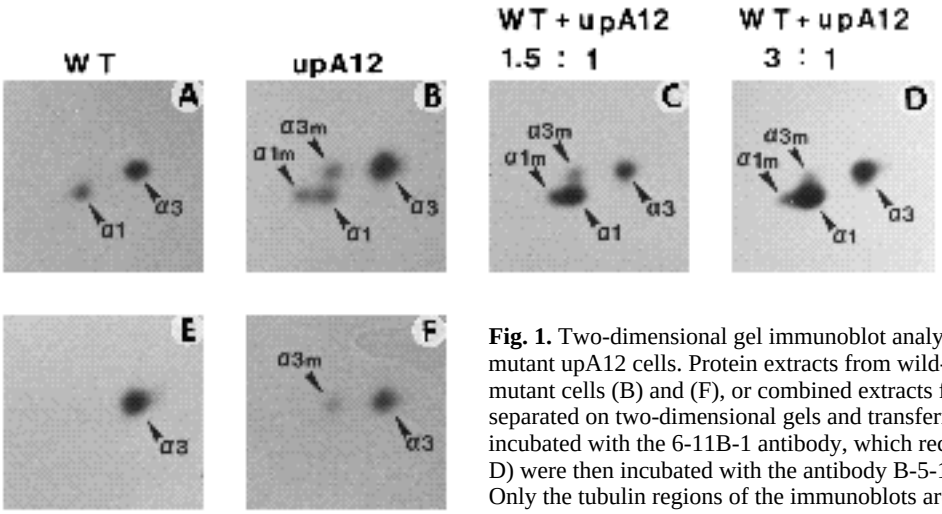

Fig. 1. Two-dimensional gel immunoblot analysis of α-tubulins in wild-type and mutant upA12 cells. Protein extracts from wild-type cells (A) and (E), from upA12 mutant cells (B) and (F), or combined extracts from both cell types (C) and (D) were separated on two-dimensional gels and transferred to nitrocellulose. The blots were incubated with the 6-11B-1 antibody, which recognizes acetylated α-tubulin. Blots (A-D) were then incubated with the antibody B-5-1-2, which recognizes all α-tubulins. Only the tubulin regions of the immunoblots are shown.

cell protein extracts. In all four tetrads, the mutant isoforms segregated 2:2 and co-segregated with APM and ORY resistance (data not shown), demonstrating that the genetic (drug-resistance) and biochemical (variant α-tubulin) phenotypes of the upA12 mutant are linked and may therefore have resulted from the same lesion.

The upA12 mutant produces two primary α-tubulin gene products

The results described above suggested that the α1 and α1m isotypes were primary gene products, but left open the possibility that α1m may have been produced by an aberrant post-translational modification of α1. To determine whether α1 and α1m are primary gene products, total RNA from wild type and mutant cells was translated in vitro in the presence of [^{35}S]methionine and the translation products were detected by autoradiography after resolution in two-dimensional gels. As previously reported (Lefebvre et al., 1980), RNA from wild-type cells stimulated the synthesis in vitro of a single α-tubulin (Fig. 2A), and the position of this gene product relative to β-tubulin was similar to that observed for α1-tubulin on two-dimensional immunoblots of whole cells or axonemes (see Fig. 3). When total RNA from mutant upA12 was translated in vitro, a labeled protein co-migrating with α1 was produced along with a slightly more basic protein not observed in translation products prepared from RNA from wild-type cells (Fig. 2B). We conclude that the α1 and α1m isoforms of mutant upA12 are primary gene products and that acetylation of each gives rise to the α3 and α3m species, respectively.

The acetylated mutant isoform (α3m) is incorporated into functional microtubules

To determine whether one or both mutant α-tubulins participate in microtubule function in vivo, the tubulin composition of mutant and wild-type flagellar axonemes was analyzed on two-dimensional immunoblots. The major α-tubulin in wild-type axonemes is the acetylated form, α3 (Lefebvre et al., 1980), although a small amount of the α1 primary protein is also present (Fig. 3A). Axonemes from mutant upA12 cells contained predominantly the acetylated isoforms α3 and α3m (Fig. 3B), demonstrating that the acetylated isoform of the mutant tubulin assembles into functional microtubules. The α1m isoform was not detected in axonemes, even in overloaded gels (Fig. 3C), indicating that this altered primary gene product is not incorporated into axonemal microtubules or is incorporated very infrequently in comparison to α1-tubulin.

The upA12 lesion is a missense mutation in the α1-tubulin gene

To determine the nature of the upA12 mutation, fragments of genomic DNA containing the α1-tubulin gene from the upA12 mutant and the NO5c^{+} wild-type strain were cloned and sequenced. With the exception of two single nucleotide differences in intron 1, the sequence of the wild-type α1-tubulin gene was identical to that published for a different strain (21gr^{+}) of *C. reinhardtii* (Silflow et al., 1985). A single T to C transition in the upA12-derived gene was the only difference observed between the sequences of the mutant and wild-type strain. This transition changed a TAC codon in the wild-type allele to a CAC in the upA12 allele

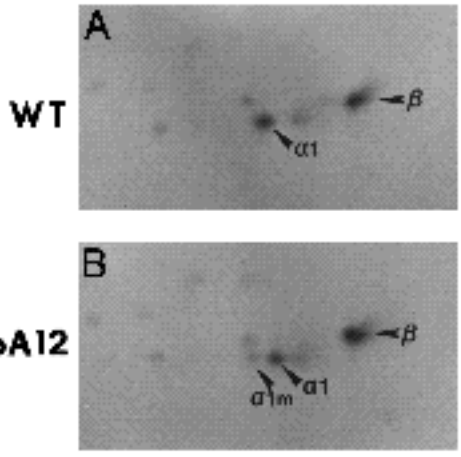

Fig. 2. Tubulin areas of autoradiograms of two-dimensional gels containing proteins translated in vitro from *Chlamydomonas* total RNA in the presence of [^{35}S]methionine. (A) Wild type; (B) upA12.

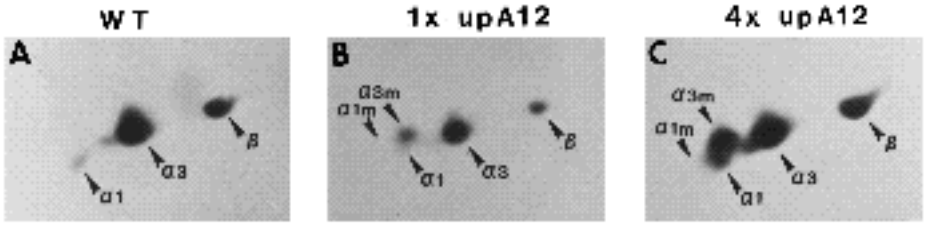

Fig. 3. Tubulin areas of two-dimensional immunoblots of axonemal proteins probed with the B512 α-tubulin and 2-10B-6 β-tubulin antisera. (A) Wild type; (B) upA12; (C) upA12, the gel was loaded with 4 times the amount of protein loaded in (B).

(Fig. 4). As a result, the upA12 α1-tubulin gene encodes a histidine residue instead of tyrosine at amino acid 24. The basic charge change predicted by the replacement of tyrosine by histidine is consistent with the mobility shift observed for the α-tubulin proteins from upA12 relative to wild type. The upA12 mutant is now designated *tua1-1* (alpha1-tubulin).

The α1- and α2-tubulin genes encode identical proteins

The simplest explanation for the α-tubulin isoforms that we observed in *tua1-1* is that the two α-tubulin genes in wild-type *Chlamydomonas* produce identical proteins, and the *tua1-1* mutation alters the charge of the protein from the α1-tubulin gene. However, previous analysis of cDNA clones corresponding to the two α-tubulin genes predicted that the protein products of the two genes differ at two amino acid residues, one of which would produce a charge difference between the two proteins (Silflow et al., 1985). To investigate the possibility of errors in the earlier DNA sequencing, we isolated genomic DNA fragments containing the α2-tubulin gene. These fragments were sequenced on one DNA strand using the same series of oligonucleotide primers used for sequencing the mutant and wild-type α1-tubulin genes. The sequencing results indicated three nucleotide positions at which the wild-type sequence (obtained from strain NO5c$^+$) differed from the published coding sequence (obtained from strain 21gr). At position 759 in the α2 gene, a C instead of a previously reported T was found. This difference occurs at the third position of a codon and does not change the predicted amino acid sequence. However, at positions 923 and 1097, a T was found instead of the G reported in the published sequence. These changes occur at the second base of a codon and predict that the α2 gene encodes an arginine at amino acid position 308 and a glycine at position 366. The corrected α2 gene sequence predicts that the α2 gene encodes a protein identical to that encoded by the α1 gene.

We also determined the partial DNA sequence of three cDNA clones corresponding to the α2-tubulin gene (obtained from a library described by Schloss et al., 1984). One of these clones, pcf4-2, was the clone used in the previous sequencing study (Silflow et al., 1985). Sequence from this clone showed a T at positions 923 and 1097, as previously reported. However, clones pcf2-18 and pcf10-13 contain a G at positions 923 and 1097, in agreement with the NO5c$^+$ genomic DNA. We conclude that the published sequence is incorrect and resulted from errors introduced into the pcf4-2 cDNA during cloning.

Mutant upA12 regenerates flagella in the presence of APM

Because the upA12 (*tua1-1*) mutation conferred resistance to APM and ORY and because the α3m isoform was incorporated into axonemal microtubules, it was of interest to study assembly of flagellar microtubules in the presence and absence of the herbicides. After deflagellation of *Chlamydomonas* cells, the two flagella regenerate rapidly, achieving full length (10-12 μm) within 60-90 minutes (Rosenbaum and Child, 1967). upA12 cells containing the *tua1-1* mutation had two flagella of wild-type length, which regenerated with essentially wild-type kinetics in the absence of the drugs (data not shown). In the presence of 0.8 μM APM, regeneration in wild-type cells did not begin until after 40 min and the flagella regenerated to approximately 25% of wild-type length after 160 min (Fig. 1A). Flagellar regeneration of upA12 mutants in APM began immediately after deflagellation and the flagella reached approximately 75% of full length after 160 min (Fig. 1B). These results demonstrate that the altered α-tubulin in cells with the *tua1-1* mutation substantially enhances assembly of axonemal microtubules in the presence of APM. Cells containing the *apm1-18* mutation also regenerated substantially better than wild-type cells in the presence of 0.8 μM APM (data not shown). Flagellar regeneration in cells containing both the *tua1-1* and *apm1-18* mutations (upA12 *apm1-18*) in the presence and absence of APM was similar to that of upA12 mutants (Fig. 5C).

DISCUSSION

We have shown that a step-up mutation isolated for its ability to synergistically enhance APM resistance with *apm1* mutations resulted from a single amino acid change in the

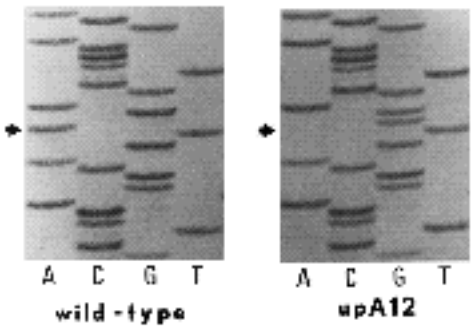

Fig. 4. Comparison of the nucleotide sequencing gels for the *C. reinhardtii* wild type (NO5c$^-$) and mutant upA12 α1-tubulin genes showing the T-A to C-G transition at codon 24. The position of the mutation is marked with an arrow.

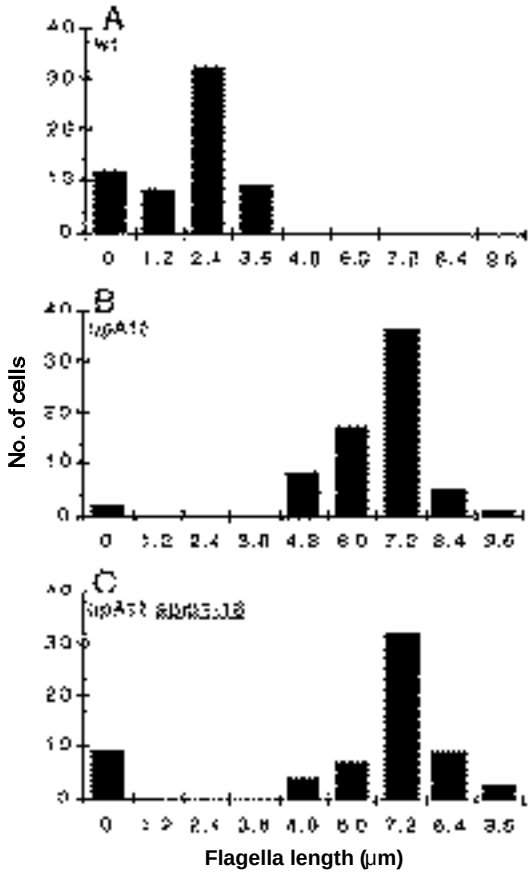

Fig. 5. Flagellar regeneration in the presence of APM. After deflagellation, cells were allowed to regenerate flagella in the presence of 0.8 μM APM. Samples of cells were removed for determination of flagellar length at various times after deflagellation; results at 160 min post-deflagellation are shown. (A) Wild type; (B) upA12; (C) upA12 *apm1-18* double mutant.

α1-tubulin gene of *Chlamydomonas reinhardtii*. The substitution of a histidine for tyrosine at amino acid 24 occurs in a hydrophobic region of ten amino acids (aa 20-30) which is highly conserved among α-tubulins from all species. Although the conservative substitution of phenylalanine for tyrosine has been observed at this position in α-tubulins predicted from gene sequences in *Trypanosoma* (Kimmel et al., 1985) and *Stylonychia* (Helftenbein, 1985) and in a divergent gene expressed in chicken testis (Pratt et al., 1987), no other substitution at this position has been reported.

As a consequence of this missense mutation, we have been able to distinguish electrophoretically the products of the two α-tubulin genes and to demonstrate several properties of the mutant and wild-type gene products. The acetylated mutant isoform (α3m) is incorporated into a functional microtubule complex (the axoneme) with α3 and α1 wild-type proteins, but the mutant primary gene product (α1m) was not detected in axonemes. This observation suggests that the α1m protein may be defective in assembly. Although no attempt was made to quantitate the levels of mutant and wild-type gene products in upA12 (*tua1-1*), the mutant α1m and α3m isoforms always appeared less abundant than the α1 and α3 isoforms in 2-D immunoblots of whole cell protein extracts. In vitro translation of total RNA from deflagellated cells produced smaller amounts of the α1m mutant isoform than of the wild-type α1 isoform. Previous comparisons of transcript levels using gene-specific hybridization probes for the two α-tubulin genes showed that the two transcripts are present at nearly identical levels in wild-type cells (Youngblom, 1987). These results suggest that transcripts of the *tua1* (α1-tubulin) gene may be translated less efficiently than transcripts from the *tua2* (α2-tubulin) gene both in vitro and in vivo. Alternatively, the defective α-tubulin produced by the *tua1-1* allele may be less stable both in vivo and in the in vitro translation mix than the wild-type α-tubulins produced by the *tua2* gene.

Strains carrying the *tua1-1* mutation are characterized by relatively weak (twofold) resistance to the plant-specific antimicrotubule drugs APM and ORY, and by twofold greater sensitivity to the microtubule-stabilizing agent TAX. Similar phenotypes involving resistance and collateral sensitivity to antimicrotubule agents have been observed for tubulin mutants in CHO cells (Schibler and Cabral, 1985a) and in *Chlamydomonas* (Schibler and Huang, 1991), where COL-resistant β-tubulin mutants were TAX-supersensitive. In addition, TAX-resistant α- and β-tubulin mutants in CHO cells are supersensitive to COL (Schibler and Cabral, 1985a). These results have been interpreted to mean that resistance or supersensitivity results from increased stability or lability of microtubules, instead of alterations in drug binding to microtubule protein. Evidence supporting this hypothesis has been obtained from studying the *col^{R}4* and *col^{R}15* β-tubulin mutations of *Chlamydomonas*, which resulted in more highly acetylated microtubules in the cytoplasm and mitotic spindle than were observed in wild-type cells (Schibler and Huang, 1991). The acetylation of α-tubulin is associated with increased stability of microtubules (Piperno et al., 1987).

Resistance to microtubule-destabilizing drugs (APM and ORY) is an unusual phenotype for α-tubulin mutants. In fungi, for example, the majority of α-tubulin mutations confer cold-sensitivity and supersensitivity to the microtubule-destabilizing benzimidazole (BEN) drugs (Toda et al., 1984; Oakley et al., 1987; Stearns and Botstein, 1988; Schatz et al., 1988). The *tua1-1* mutation is similar to α-tubulin mutants of CHO cells selected for resistance to maytansine (MAY) and subsequently shown to confer supersensitivity to TAX (Schibler and Cabral, 1985b). Both upA12 and the MAY^r α-tubulin mutants express resistance semi-dominantly, but upA12 differs from the MAY^r mutants because the CHO mutants are cross-resistant to COL, whereas upA12 exhibits wild-type sensitivity to this anti-microtubule drug.

With respect to COL resistance, the upA12 mutation differs from the β-tubulin mutants of *Chlamydomonas*, which display resistance to a variety of antimicrotubule agents, including COL, APM and ORY (Bolduc et al., 1988; Schibler and Huang, 1991). It is not clear why upA12 should be COL-sensitive. However, these observations for the *Chlamydomonas* α- and β-tubulin mutations are consistent with the findings that most COL-resistant tubulin

mutants in CHO cells are in β-tubulin (Cabral et al., 1980) and BEN-resistant tubulin mutants of *Aspergillus* (Oakley and Morris, 1980; Oakley and Morris, 1981) and *Saccha - romyces cerevisiae* are exclusively in β-tubulin (Huffaker et al., 1988; Schatz et al., 1988).

The demonstration of synergistic enhancement of APM resistance by *tua1-1* and *apm1* alleles suggests that the *apm1* gene product may be involved in microtubule-related processes, but does not indicate whether the *apm1* product interacts directly with microtubules or with microtubule-containing structures. The step-up selection described in this study was performed on a small scale, but the outcome suggests that step-up procedures may be generally useful for identifying new genes whose products may function in microtubule-related processes.

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