The col^R4 and col^R15 β-Tubulin Mutations in *Chlamydomonas reinhardtii* Confer Altered Sensitivities to Microtubule Inhibitors and Herbicides by Enhancing Microtubule Stability

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Abstract. The col^R4 and col^R15 β2-tubulin missense mutations for lysine-350 in *Chlamydomonas reinhardtii* (Lee and Huang, 1990) were originally isolated by selection for resistance to the growth inhibitory effects of colchicine. The col^R4 and col^R15 mutants have been found to be cross resistant to vinblastine and several classes of antimitotic herbicides, including the dinitroanilines (oryzalin, trifluralin, profluralin, and ethafluralin); the phosphoric amide amiprophos methyl; and the dimethyl propynl benzamide pronamide. Like colchicine and vinblastine, the antimitotic effects of these plant-specific herbicides have been associated with the depolymerization of microtubules. In contrast to their resistance to microtubule-depolymerizing drugs, the mutants have an increased sensitivity to taxol, a drug which enhances the polymerization and stability of microtubules. This pattern of altered sensitivity to different microtubule inhibitors was found to cosegregate and corevert with the β-tubulin mutations providing the first genetic evidence that the in vivo herbicidal effects of the dinitroanilines, amiprophos methyl, and pronamide are related to microtubule function. Although wild-type like in their growth characteristics, the col^R4 and col^R15 mutants were found to have an altered pattern of microtubules containing acetylated α-tubulin, a posttranslational modification that has been associated with stable subsets of microtubules found in a variety of cells. Microtubules in the interphase cytoplasm and those of the intranuclear spindle of mitotic cells, which in wild-type *Chlamydomonas* cells do not contain acetylated α-tubulin, were found to be acetylated in the mutants. These data taken together suggest that the col^R4 and col^R15 missense mutations increase the stability of the microtubules into which the mutant β-tubulins are incorporated and that the altered drug sensitivities of the mutants are a consequence of this enhanced microtubule stability.

In both animal and plant cells microtubules comprise a major component of the cytoskeleton and are involved in several vital cellular functions including nuclear division, several forms of cell motility, transport of organelles, cell shape determination, and cytoplasmic rearrangement during the cell cycle (Snyder and McIntosh, 1975; Dustin, 1984; Schliwa, 1984). Studies on microtubule function and assembly have been facilitated by the use of a wide variety of inhibitor drugs which block microtubule-dependent processes in cells by binding to the tubulin dimers or polymerized microtubules. The in vivo mechanism of action of most of the microtubule inhibitors identified to date is to depolymerize microtubules and to inhibit their assembly. Microtubule-depolymerizing drugs include the plant alkaloids such as colchicine, vinblastine, podophyllotoxin, and maytansine, the antifungal and antihelminthic benzimidazole derivatives, and several plant-specific antimitotic herbicides including the dinitroanilines trifluralin and oryzalin and the phosphoric amide herbicide amiprophos-methyl (for review see Morejohn and Fosket, 1986). Unique among the microtubule inhibitors is the taxane alkaloid, taxol, which inhibits microtubule-dependent processes by stabilizing microtubules and promoting microtubule polymerization (Schiff et al., 1979, Schiff and Horwitz, 1980, Parness and Horwitz, 1981).

One approach to the study of microtubule function in which microtubule inhibitor drugs has been particularly useful has been in the isolation of mutations in the structural genes for α- and β-tubulin. Over the past 10 yr tubulin mutants have been isolated on the basis of altered sensitivity to the growth-inhibitory effects of microtubule inhibitors in a number of different organisms including mammalian cells in culture and various fungi such as *Aspergillus*, *Neurospora*, *Physarum*, and the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (for a review see Cabral, 1989).

In previous studies from this laboratory, two β-tubulin mutants in the unicellular green alga *Chlamydomonas reinhardtii* were isolated on the basis of resistance to the growth

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inhibitory effects of colchicine (Bolduc et al., 1988). These mutants, *col*^{R}*4* and *col*^{R}*15*, expressed unique variant β-tubulin isoforms which were found assembled into cellular microtubules. In wild-type *Chlamydomonas*, there are two β-tubulin genes which encode identical proteins (Youngblom et al., 1984). The *col*^{R}*4* and *col*^{R}*15* mutations have been shown to be missense mutations for the amino acid residue lysine-350 in β2-tubulin (Lee and Huang, 1990).

In preliminary studies, it was determined that in addition to colchicine resistance, both mutants expressed altered sensitivity to a number of other microtubule inhibitors including several plant-specific antimitotic herbicides. To better understand the mechanism by which these missense mutations affect microtubule structure and function, we have examined in detail the pattern of drug sensitivity of the mutants, the genetic segregation and reversion of the mutant phenotypes, and the effects of the mutations on the in vivo biochemical properties of the cellular microtubules. The results of these analyses suggest that the assembly-competent *col*^{R}*4* and *col*^{R}*15* mutant β2-tubulins confer altered sensitivities to specific microtubule inhibitors and herbicides by enhancing the stability of the microtubules into which they are assembled. The herbicide-resistant phenotypes of the mutants provide the first genetic evidence that the in vivo herbicidal effects of the dinitroanilines, APM, and pronamide are related to microtubule function.

Materials and Methods

Materials

Colchicine, vinblastine, nocodazole (Methyl[5-(2-thienylcarbonyl)-1H-benzimidazol-2-yl] carbamate), griseofulvin, and podophyllotoxin were obtained from Sigma Chemical Corp. (St. Louis, MO). Taxol was generously supplied by Dr. M. Suffness at the Developmental Therapeutics Branch of the National Cancer Institute (Bethesda, MD). Oryzalin, trifluralin, ethafluralin, profluralin, benfluralin, terbutol, and dimethyltetrachlorterephthalate (DCPA)[1] and pronamide were obtained from Chem Service, Inc. (West Chester, PA). Amiprophos-methyl (APM) was a gift from Mobay Chemical Corp. (Pittsburgh, PA). Isopropyl-*N*-phenyl-carbamate (IPC) was obtained from PPG Industries, Inc., (Pittsburgh, PA).

The mouse monoclonal anti-α-tubulin antibody 4.22.6 used in this study was raised in this laboratory against the protein isolated from *Chlamydomonas* and reacts with all species of α-tubulin present in the cell. The other mouse mAb 6-11B-1 was the gift of Dr. G. Piperno (Rockefeller University, New York) and was raised against tubulin from the axonemes of sea urchin sperm flagella and recognizes acetylated α-tubulin in a variety of organisms (Piperno and Fuller, 1985).

Strains, Cell Culture, and Genetic Analysis

Wild-type strains of *Chlamydomonas reinhardtii 137c mt*$^{+}$ and *mt*$^{-}$ were obtained from Dr. E. Harris at the *Chlamydomonas* Genetics Center at Duke University, Durham, NC. Strains *col*^{R}*4* and *col*^{R}*15* were isolated from *137c mt*$^{+}$ and characterized as previously described (Bolduc et al., 1988). Cells were grown in Tris-acetate-phosphate (TAP) medium (Gorman and Levine, 1965) with 24-h illumination and agitation at 25°C or on TAP agar plates with 24-h illumination at 20°C. Standard techniques of crossing and tetrad analysis were used to determine segregation patterns (Levine and Ebersold, 1960).

Spontaneously arising taxol-resistant revertants of *col*^{R}*4* and *col*^{R}*15* were selected from 10 separate isolates of each strain. Approximately 5×10^5 cells per plate were spread onto TAP plates containing 6×10^{-6} M taxol and grown in constant light at 25°C for 7–10 d. Several resistant colonies were picked and streaked onto plates containing the same concentration of the drug.

Drug Treatments

A simple assay for growth in liquid culture was used based on the measurement of chlorophyll content of cells in wells of 96-well microtiter dishes by light absorbance after lysis. A plot of cell density vs. light absorbance at 660 nm was linear (Fig. 1) and all measurements of cell growth in liquid culture fell within the linear range. For measurement of growth inhibition by various drugs, 2×10^4 cells were seeded in wells of a 96-well microtiter dish containing 200 μl of TAP medium plus or minus various concentrations of drug and were grown in constant light at 20°C for 4 d. The cells were lysed by adding 50 μl of a solution containing sodium dodecyl sulfate (Laemmli, 1970) to the wells. Light absorbance at 660 nm was read on an ELISA plate reader within 10 min of lysis and plotted vs. drug concentration for each strain.

Immunofluorescence

The preparation of fixed *Chlamydomonas* cells and the protocol for indirect immunofluorescence analysis was performed as previously described (Huang et al., 1988). The cells were double labeled with two mouse mAbs directed against total and acetylated α-tubulin based on a previously described procedure (LeDizet and Piperno, 1986). The sequence of antibody incubations was as follows: (*a*) the mouse mAb 6-11B-1 directed against acetylated α-tubulin (Piperno and Fuller, 1985) diluted 1:50 in PBS; (*b*) a sheep antimouse antibody conjugated with biotin (Amersham Corp., Arlington Heights, IL) diluted 1:50 in PBS; (*c*) the mouse mAb 4.22.6 against total α-tubulin (as harvest fluid) diluted 1:50; and (*d*) a solution containing streptavidin conjugated with Texas red (final dilution of 1:50) (Amersham Corp.) and FITC-conjugated antimouse IgG antibody diluted 1:20 (Fisher Biotech, Orangeburg, NY). The cells were incubated in each of the antibody solutions for 30–45 min at 37°C. After each incubation, the slides were rinsed in PBS and blocked in a solution containing 0.1% BSA and 0.01% NaN_3. The slides were mounted as previously described (Huang et al., 1988) and viewed and photographed on an Axiophot (Zeiss, Oberkochen, Germany) or an inverted microscope (model IM35; Zeiss) equipped with epifluorescence optics.

Protein Extraction and Determination

For isolation of whole cell body proteins, cells were grown on TAP agar plates and deflagellated by pH shock as previously described (Huang et al., 1979), centrifuged as described above and supernatant (containing flagella) discarded. Pellets were resuspended in hot SDS buffer (Laemmli, 1970) (1 ml per 10^8 cells). PMSF (Sigma Chemical Co.) was added to each suspension to a final concentration of 1 mM. The suspensions were boiled for 2 min, transferred to 30-ml COREX tubes, and centrifuged at room temperature at 10,000 rpm in a rotor (model JA-20; Beckman Instruments, Inc., Palo Alto, CA) in a centrifuge (model J2-21; Beckman Instruments, Inc.)

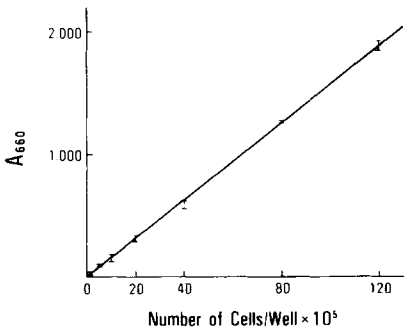

Figure 1. Assay for growth of *Chlamydomonas* in liquid culture based on the chlorophyll content of the cells. Various numbers of cells were plated in final volumes of 200 μl of TAP medium in wells of a 96-well microtiter dish. They were then lysed by adding 50 μl of sodium dodecyl phosphate sample buffer (Laemmli, 1970) to the wells; light absorbance at 660 nm was read on an ELISA plate reader and plotted vs. number of cells.

1. *Abbreviations used in this paper*: APM, amiprophos-methyl; DCPA, dimethyltetrachlorterephthalate; IPC, isopropyl-*N*-phenyl-carbamate; TAP, Tris-acetate-phosphate.

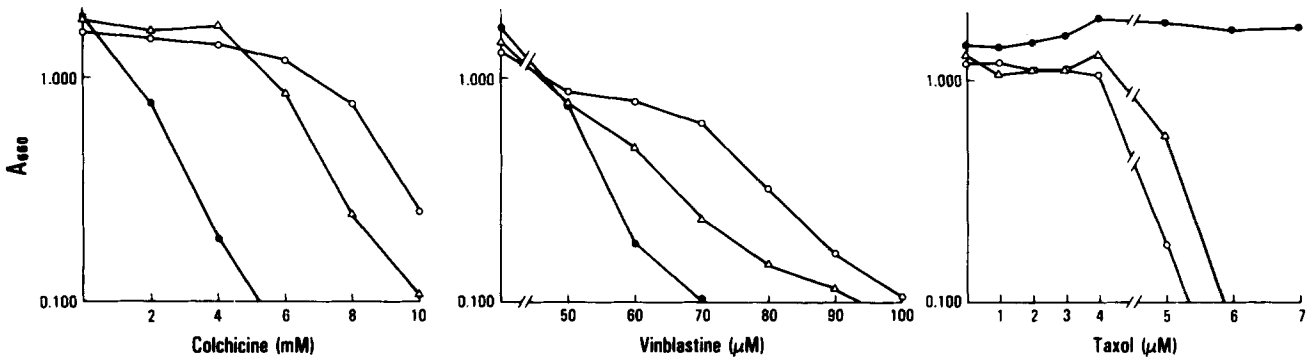

Figure 2. Growth inhibition of wild-type parental, *col*^{R}*4*, and *col*^{R}*15* cells in the presence of colchicine, vinblastine, and taxol. 2×10^4 cells were seeded in wells of a 96-well microtiter dish containing 200 μl of Tap medium plus or minus various concentrations of drug and were grown in constant light at 20°C for 4 d. They were then lysed as for Fig. 1 and absorbance at 660 nm was read and plotted vs. drug concentration for each strain. The final points for each cell type below an absorbance of 0.1 were plotted, but are not shown. (●), Wild-type; (△), *col*^{R}*4*; (○), *col*^{R}*15*.

for 15 min. Supernatants were aliquoted and frozen at −70°C for gel samples. For protein determinations, aliquots were removed and 5 vol of ice-cold acetone were added to extract chlorophyll which interfered with protein assays. Samples were spun in a microfuge for 10 min, the resulting pellets resuspended in distilled H_2O, and protein concentrations were determined with the BCA protein assay (Pierce Chemical Co., Rockford, IL).

Flagellar axonemes were isolated and solubilized as previously described (Bolduc et al., 1988).

PAGE and Immunoblot Analysis

Chlamydomonas deflagellated whole cell body proteins were resolved on 9% polyacrylamide gels containing SDS as previously described (Laemmli, 1970). The proteins were electrophoretically transferred onto 0.2 μm pore-size nitrocellulose according to Towbin et al. (1979) for 4 h with a voltage gradient of 4.5 V/cm. The blots were blocked for 1 h in TBS (50 mM Tris, 0.2 M NaCl, pH 7.4) containing BLOTTO (Johnson et al., 1984), then incubated for 1 h or overnight in primary antibody as harvest fluids diluted in BLOTTO at 1:100 for 4.22.6 and 1:15 for 6-11B-1. After being washed in TBS, the blots were incubated overnight in a solution of ^{125}I-conjugated antimouse Ig (100 μCi/ml; sp. act. 19 Ci/μg; Amersham Corp.) diluted in BLOTTO. After extensive washing in several changes of TBS, the blots were dried and exposed to x-ray film (XAR-5; Eastman Kodak Co., Rochester, NY).

Chlamydomonas flagellar axonemal proteins were resolved on slab isoelectric focusing gels and the identity of the β-tubulins determined as previously described (Bolduc et al., 1988).

Results

col^{R}4 and col^{R}15 Mutants Are Cross Resistant to Vinblastine and Microtubule Disrupting Herbicides, But Are Sensitive to Taxol

Wild-type, *col*^{R}*4*, and *col*^{R}*15* strains were initially tested for their ability to grow in liquid cultures in the presence of several known microtubule inhibitors and antimitotic herbicides. The inhibitors fall into two categories: first, those known to affect microtubules and microtubule related processes in animal cells, and second, herbicides which have been reported to inhibit mitosis- or microtubule-related processes specifically in plant cells. Among the drugs we tested which have been shown to be effective mitotic and microtubule inhibitors at micromolar or lower concentrations in animal cells, only colchicine and vinblastine inhibited growth of wild-type *Chlamydomonas* cells. Podophyllotoxin, griseofulvin, nocodazole, and taxol had no effect on wild-type cells at any concentration up to the limits of their solubility in the TAP culture medium. Among the herbicides we tested, wild-type cells were growth inhibited by pronamide, APM, IPC, and the dinitroanilines oryzalin, trifluralin, profluralin, and ethafluralin. The herbicides benfluralin, Terbutol (Vaughn et al., 1987), and DCPA (Holmsen and Hess, 1985) did not inhibit growth of wild-type cells up to the limits of their solubility in the TAP culture medium.

In addition to being resistant to colchicine at higher concentrations than wild-type cells, both *col*^{R} strains were cross resistant in liquid culture to higher levels of vinblastine than wild-type cells (Fig. 2). The mutants were resistant to a two-fold higher concentration of colchicine and a 1.5-fold higher concentration of vinblastine than wild type cells. In the presence of the microtubule stabilizing drug taxol, the growth properties of the cells were the reverse of those observed with colchicine and vinblastine. Wild-type cells grew in taxol-containing medium up to the concentration where the drug was no longer soluble in the TAP medium (~10 μM, Fig. 2). Growth of the mutants was inhibited by taxol at concentrations of 5 μM and greater (Fig. 2).

Bcth *col*^{R} mutants were also resistant to higher concentrations of the herbicides pronamide, APM, and the dinitroanilines oryzalin, trifluralin, profluralin, and ethafluralin than wild-type cells. The dose responses of the two mutants as well as the wild-type cells to growth in the presence of pronamide, APM, and oryzalin are shown in Fig. 3. Both mutants were resistant to a 10-fold greater concentration of pronamide and two- to threefold higher levels of APM and oryzalin than wild-type cells. In contrast, the *col*^{R} mutants expressed a sensitivity to IPC paralleling that observed in wild-type (data not shown). The observation that the *col*^{R}*4* and *col*^{R}*15* mutations do not affect the concentration of IPC required to inhibit growth are consistent with the observation that IPC disrupts mitosis not by affecting microtubule assembly, but rather the organization of the microtubules. In a number of different plant cells IPC has been shown to produce multipolar mitoses leading to the suggestion that the drug disrupts the normal function and/or disposition of microtubule-organizing centers rather than microtubules themselves (Hepler and Jackson, 1969; Coss et al., 1975; Doonan et al., 1985; Clayton and Lloyd, 1984).

As has been previously described (Walne, 1966), wild-type *Chlamydomonas* cells grown in the presence of 5 mM

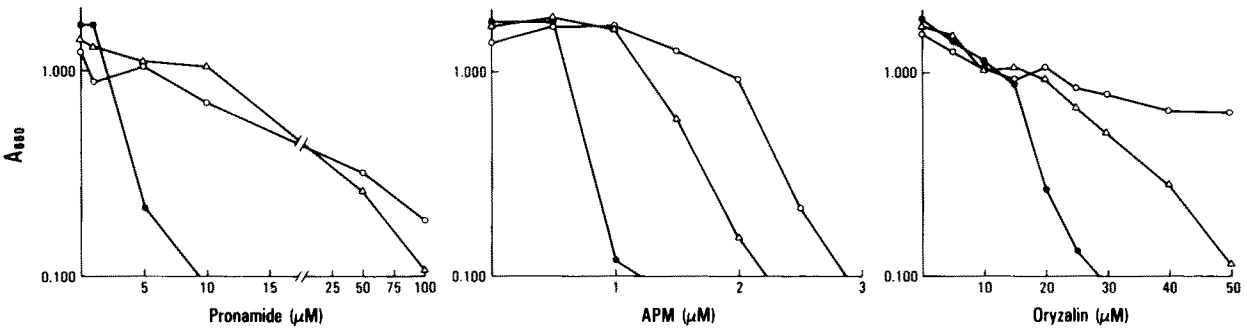

Figure 3. Growth inhibition of wild-type parental, *col*^{R}*4*, and *col*^{R}*15* cells in the presence of pronamide, amiprophos methyl (*APM*), and oryzalin. Cells were grown and treated as in Fig. 2. Final points are not shown. (●), Wild-type; (△), *col*^{R}*4*; (○), *col*^{R}*15*.

colchicine become greatly enlarged and show an absence of assembled flagella (Fig. 4). In contrast, *col*^{R}*4* and *col*^{R}*15* mutants grown in the presence of 5 mM colchicine are generally flagellated and normal in size (Fig. 4). The respective appearances of both wild-type and *col*^{R} mutant cells after growth in the presence of pronamide or APM mimic those of the same cells grown in colchicine (Fig. 4). When grown in the presence of 6 μM taxol, the cytological appearances of wild-type and mutant cells are reversed (Fig. 4).

The col^R*4 and col*^{R}*15 Altered Drug Sensitivity Phenotypes Cosegregate and Corevert with the β-Tubulin Mutations*

The colchicine-resistant phenotypes of *col*^{R}*4* and *col*^{R}*15* were previously shown to cosegregate in genetic crosses with the presence of two different acidic variant β-tubulins (Bolduc et al., 1988). To verify that the altered sensitivities of the mutants to the other microtubule inhibitors were genetically linked to the β-tubulin mutations, *col*^{R}*4* and *col*^{R}*15* strains were crossed with wild-type and the cloned meiotic progeny from at least 10 complete tetrads from each cross were scored for growth in the presence of 5 mM colchicine, 60 μM vinblastine, 6 μM taxol, 10 μM pronamide, 1 μM APM, and 20 μM oryzalin. All daughter tetrads segregated 2:2 for sensitivity and resistance to 5 mM colchicine. Invariantly, all colchicine-resistant daughter cells grew in the presence of vinblastine, pronamide, APM, and oryzalin but were growth inhibited by taxol. Conversely, all colchicine-sensitive cells were sensitive to vinblastine and the herbicides, but resistant to taxol.

The *col*^{R}*4* and *col*^{R}*15* altered drug sensitivity phenotypes were found not only to cosegregate in genetic crosses, but also to corevert with reversion of the mutant β-tubulin gene products to a wild-type electrophoretic mobility. Using the taxol-sensitive phenotypes of the mutants, spontaneous revertants to taxol resistance were isolated. In addition to a wild-type resistance to taxol, each of the revertants expressed a wild-type sensitivity to colchicine, vinblastine, and the different herbicides. Among these revertants, several were found in which the wild-type isoelectric focusing pattern of β-tubulin was restored. Fig. 5 shows the one-dimensional resolution by isoelectric focusing of the β-tubulin subunits found in the axonemes of wild-type, *col*^{R}*4*, *col*^{R}*15*, and two independent revertants of *col*^{R}*4* (*^{R}4D10* and *^{R}4H6*) and *col*^{R}*15* (*^{R}15B1* and *^{R}15D4*). As previously demonstrated (Bolduc et al., 1988), wild-type axonemes contain a single β-tubulin isoform while the *col*^{R}*4* and *col*^{R}*15* axonemes contain two β-tubulins, one which comigrates with the wild-type isoform and another which is more acidic. The more acidic variant β-tubulins in *col*^{R}*4* and *col*^{R}*15* have been shown to be the products of missense mutations in the β2-tubulin gene (Lee and Huang, 1990). As shown in Fig. 5, in the axonemes isolated from the revertants *^{R}4D10*, *^{R}4H6*, *^{R}15B1*, and *^{R}15D4*, only a single β-tubulin wild-type isoform is seen.

Genetic evidence was obtained that each of these revertants represented secondary mutations in the β2-tubulin gene. The revertants were backcrossed to wild-type and at least 20 complete tetrads from each cross were dissected and daughters tested for growth in 6 μM taxol and 5 mM colchicine. Resistance to 6 μM taxol and sensitivity to 5 mM colchicine segregated 4:0 in all cases, providing evidence that the revertants represented secondary intragenic mutations. These data of cosegregation and coreversion of phenotypes provide strong genetic evidence that the altered drug sensitivities expressed by the *col*^{R}*4* and *col*^{R}*15* mutants are a consequence of the β2-tubulin mutations.

col^R*4 and col*^{R}*15 Mutants Have an Altered Pattern of Cellular Microtubules Containing Acetylated α-Tubulin*

In an initial analysis of the phenotypes of *col*^{R}*4* and *col*^{R}*15*, it was observed that although the mutations conferred an increased resistance to the inhibitory effects of colchicine on multiple cellular functions including vegetative growth, flagellar assembly, and the germination of meiotic products, the mutants had wild-type growth characteristics when grown in the absence of colchicine (Bolduc et al., 1988). We have, however, detected a distinct alteration in the mutants in the distribution of cellular microtubules containing acetylated α-tubulin. The *col*^{R}*4* and *col*^{R}*15* mutants and wild-type cells grown in the absence of microtubule inhibitors were examined by indirect immunofluorescence staining using two mAbs raised against α-tubulin; one which recognizes both acetylated and nonacetylated α-tubulin (4.22.6) and one which recognizes only acetylated α-tubulin (6-11B-1). The pattern of microtubule fluorescence seen with the 4.22.6 antibody in wild-type and both *col*^{R}*4* and *col*^{R}*15* mutant cells was very similar and paralleled that previously reported for the distribution of microtubules in interphase wild-type *Chlamydomonas* cells (Doonan and Grief, 1987). In addition

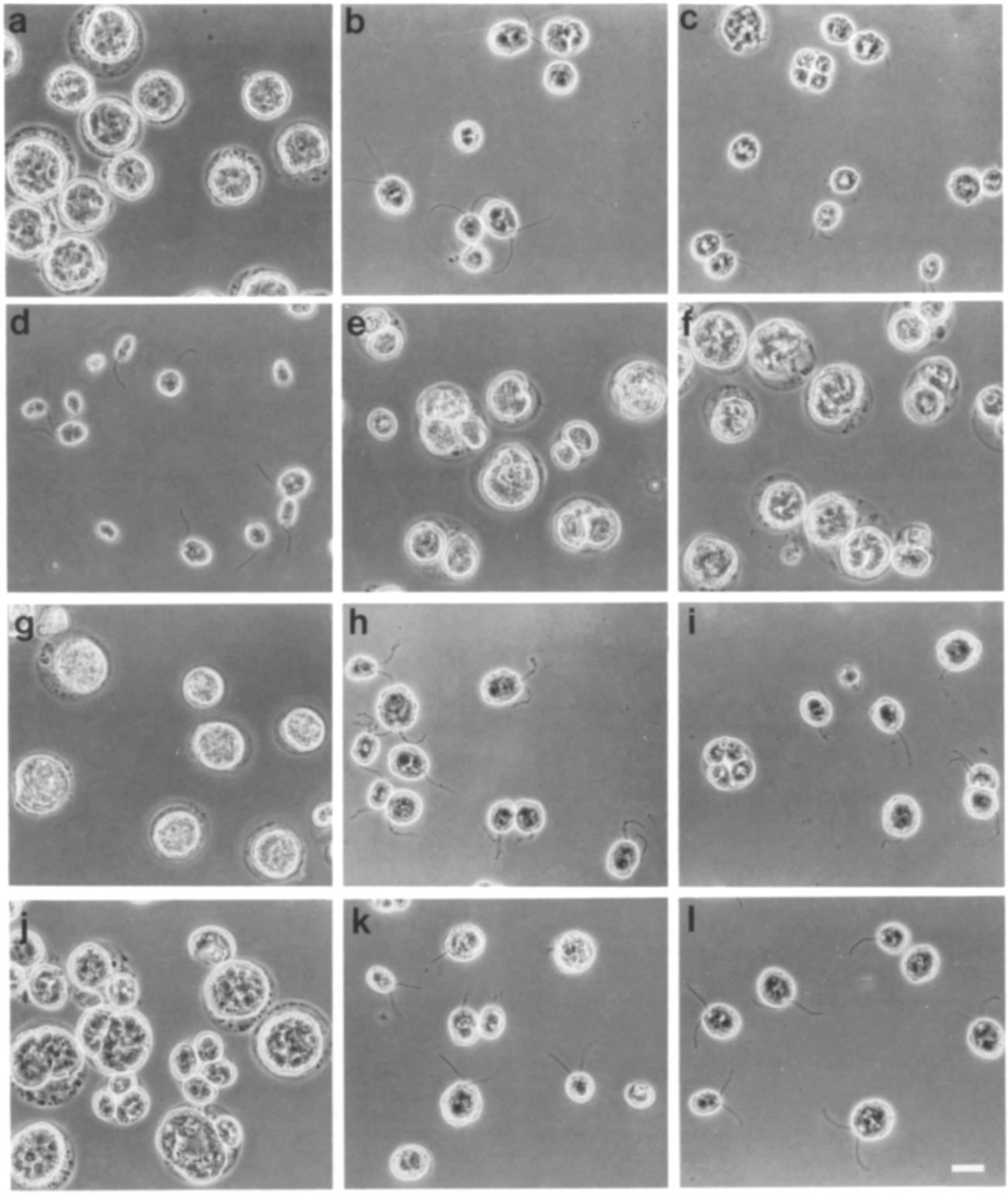

Figure 4. Morphologies of *Chlamydomonas* cells grown in the presence of different microtubule inhibitors. (*a*, *d*, *g*, *i*), wild-type; (*b*, *e*, *h*, *k*), col^R4; (*c*, *f*, *i*, *l*), col^R15; (*a*, *b*, *c*), 5 mM colchicine; (*d*, *e*, *f*), 6 μm taxol; (*g*, *h*, *i*), 10 μm pronamide; (*j*, *k*, *l*), 1 μm APM. Phase optics. Bar, 10 μm.

to the two flagella, an extensive cortical array of microtubules extending distally from the basal apparatus for approximately three quarters the length of the cell bodies was observed (Fig. 6, *a–c*). In a through focus analysis of a number of different individual cells, there appeared to be as many as 40–50 microtubules comprising this cortical array. In the same cells labeled with antibody 6-11B-1 which recognizes only acetylated α-tubulin, there was a striking difference be-

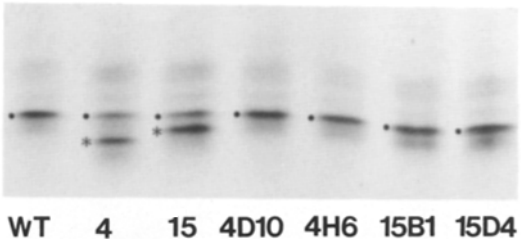

Figure 5. Tubulin region of a Coomassie blue–stained slab isoelectric focusing gel of axonemal proteins of colchicine resistant mutants and taxol-resistant revertants of *C. reinhardtii*. (*WT*), wild-type; (*4*) *col*^{R}*4*; (*15*), *col*^{R}*15*; (*4D10* and *4H6*), revertants of *col*^{R}*4*; (*15B1* and *15D4*), revertants of *col*^{R}*15*. The electrophoretic mobilities of wild-type β-tubulin (●), and the *col*^{R}*4* and *col*^{R}*15* variant β-tubulins (*) correspond to those previously described and verified by anti-β-tubulin antibody staining (Bolduc et al., 1988; Lee and Huang, 1990). The gel is oriented with the acidic end at the bottom.

tween the staining patterns of wild-type and the mutants (Fig. 6, *d–f*). In wild-type cells, only the flagella and 4 distinct bands of microtubules in the cytoplasm were stained (Fig. 6 *d*). In contrast, in both of the mutants a large number of additional microtubules in the cytoplasm were found to be stained by the anti-acetylated α-tubulin antibody (Fig. 6, *e* and *f*).

In *Chlamydomonas*, there are four microtubule rootlets consisting of cross-linked sets of microtubules which originate in the region of the basal body apparatus and extend distally (Ringo, 1967; Goodenough and Weiss, 1978). It is likely, based on their number and distribution that the four bands of microtubules stained in wild-type cells with anti-acetylated α-tubulin antibody correspond to the cross-linked microtubule rootlets.

The origin and distribution of the large numbers of additional microtubules visualized here and in an earlier report (Doonan and Grief, 1987) have not previously been defined at the ultrastructural level. In a thin-section electron microscopic analysis of fixed wild-type cells, we have obtained evidence that these microtubules are assembled onto the microtubule rootlets. Fig. 7 is a thin-section electron microscopic image through the apical region of a wild-type cell. Portions of the microtubule rootlets are visible and along their lengths, individual microtubules are seen (*arrowheads*), some of which appear to be attached at their proximal ends to the lateral surfaces of the microtubule roots (*arrows*).

When *Chlamydomonas* enters mitosis, the flagella and the majority of cytoplasmic microtubules are disassembled and an intranuclear spindle is formed (Johnson and Porter, 1968; Doonan and Grief, 1987). The interphase microtubule rootlets persist and constitute the previously described metaphase band of microtubules (Gaffal and el-Gammal, 1990). Fig. 8, *a* and *d* show double-label images of a wild-type cell in mitosis. With the antibody 4.22.6 against total α-tubulin, the intranuclear spindle as well as the metaphase band of cytoplasmic microtubules are stained (Fig. 8 *a*). In wild-type cells, the antibody 6-11B-1 specific for acetylated α-tubulin stains only the metaphase band (Fig. 8 *d*). In contrast, in both *col*^{R}*4* and *col*^{R}*15* mutant cells, many of the intranuclear spin-

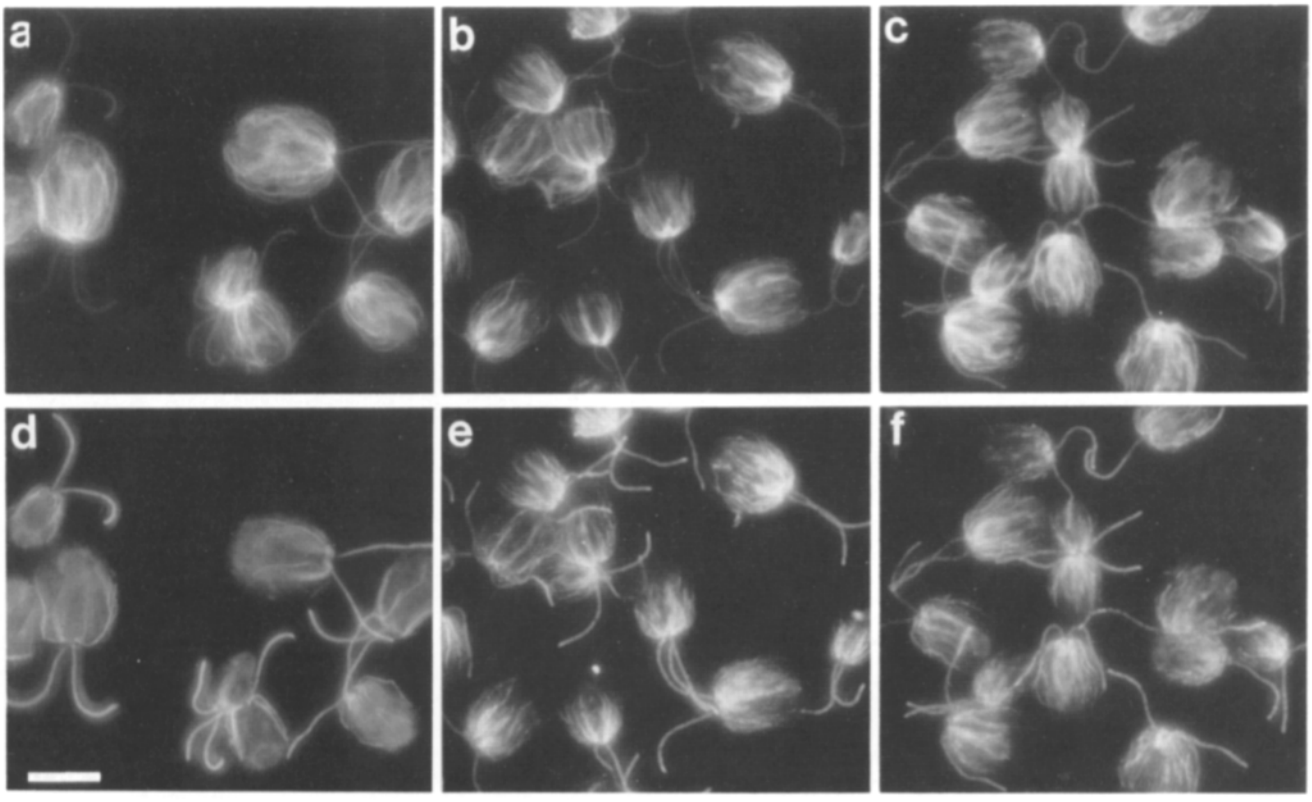

Figure 6. Double-label indirect immunofluorescence of interphase wild-type, *col*^{R}*4*, and *col*^{R}*15* cells with anti-α-tubulin mouse mAbs. (*a* and *d*), wild-type cells; (*b* and *e*), *col*^{R}*4* cells; (*c* and *f*), *col*^{R}*15* cells. (*a–c*) FITC-stained cells labeled with antibody 4.22.6 against total α-tubulin. (*d–f*), Texas red–stained cells labeled with antibody 6-11B-1 against acetylated α-tubulin. Bar, 10 μm.

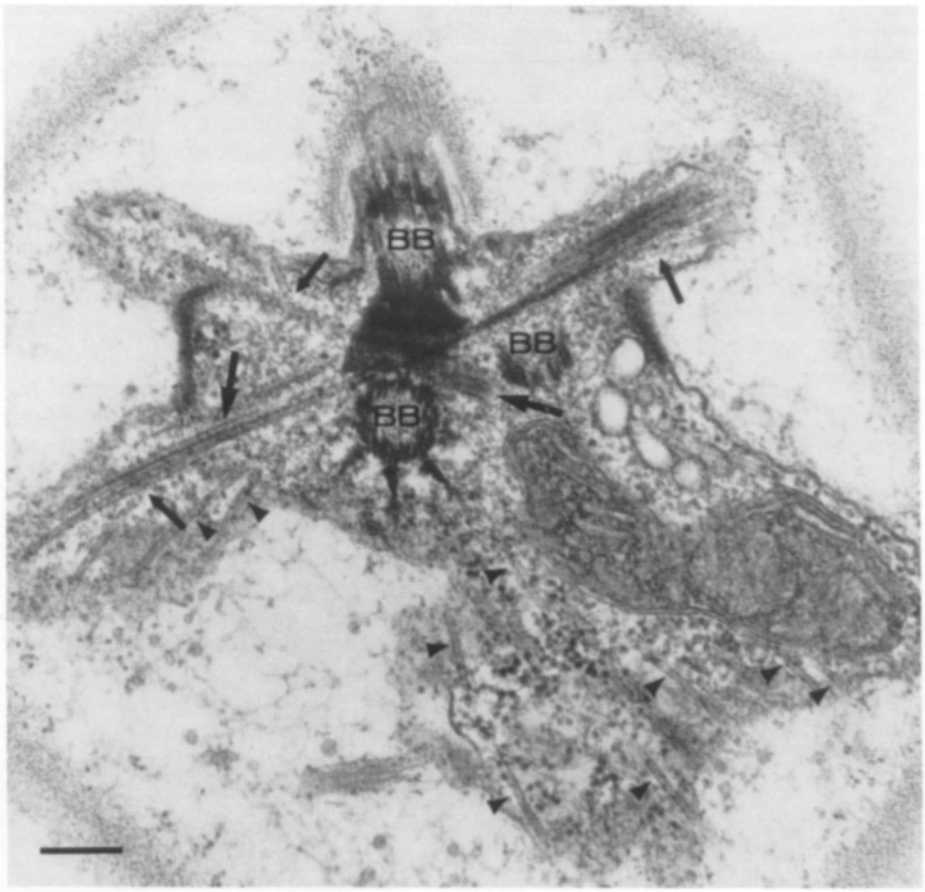

Figure 7. Electron micrograph of a transverse section through the base of the flagella in a wild-type cell showing the arrangement of the basal bodies (*BB*), rootlet microtubules (*large arrows*), and cytoplasmic microtubules (*arrowheads*). Small arrows indicate points at which cytoplasmic microtubules meet rootlet microtubules. Bar, 0.2 μm.

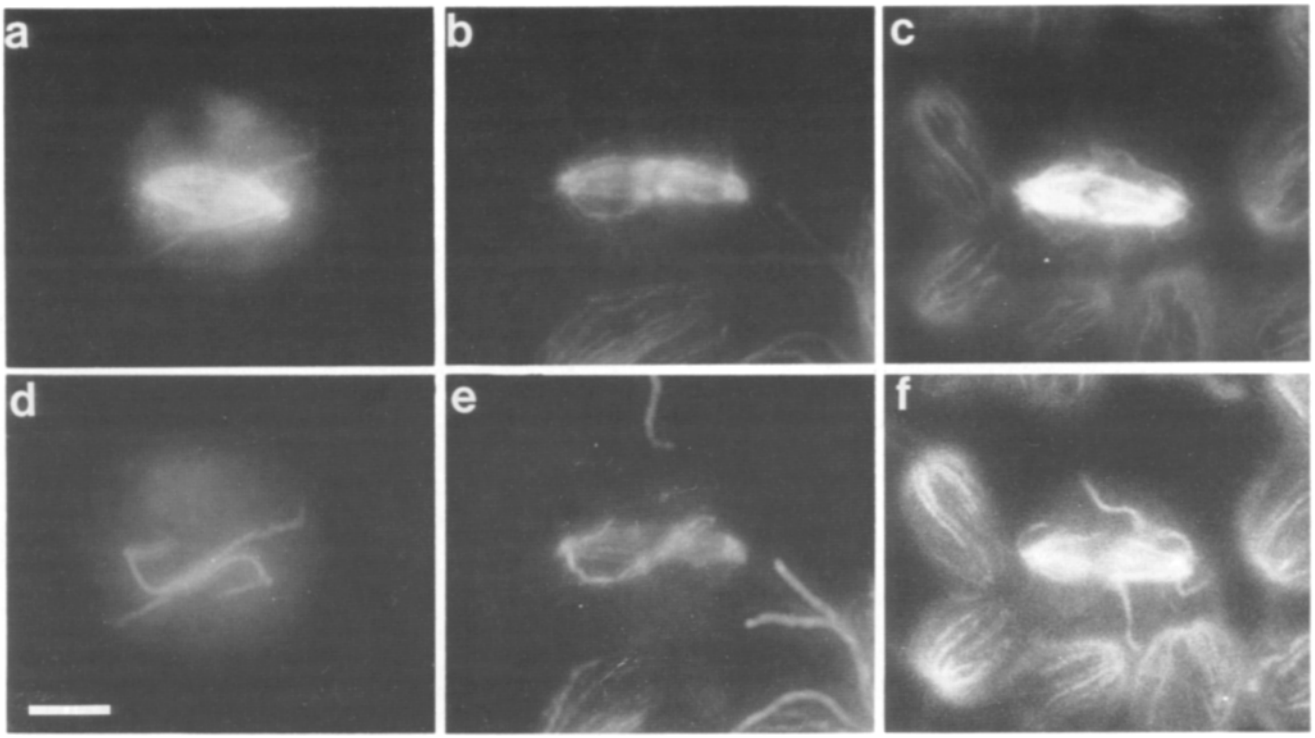

Figure 8. Double-label indirect immunofluorescence of mitotic spindles of wild-type, *col^{R}4*, and *col^{R}15* cells with anti-α-tubulin mouse mAbs. (*a* and *d*), wild-type cells; (*b* and *e*), *col^{R}4* cells; *c* and *f*, *col^{R}15* cells. (*a–c*) FITC-stained cells labeled with antibody 4.22.6 against total α-tubulin. (*d–f*), Texas red–stained cells labeled with antibody 6-11B-1 against acetylated α-tubulin. Bar, 5 μm.

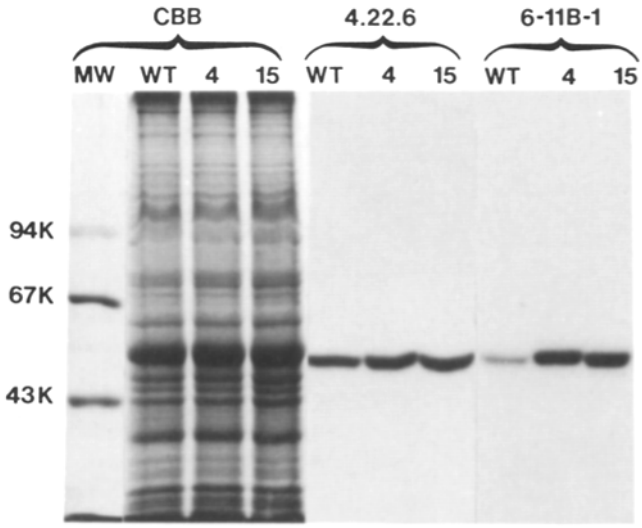

Figure 9. Coomassie blue stained gels and immunoblots of proteins of deflagellated whole cells of wild-type (*WT*), *col*^{R}*4* (*4*), and *col*^{R}*15* (*15*). Protein samples were prepared as stated in Materials and Methods and equal amounts were loaded onto 9% SDS gels. Gels were electroblotted onto nitrocellulose filters and the filters were incubated with antibody against total α-tubulin (*4.22.6*) or against acetylated α-tubulin (*6-11B-1*) followed by incubation with ^{125}I-conjugated antimouse antibody. Filters were then dried and autoradiographed. (*MW*), molecular weight standards.

dle microtubules are also stained with antibody 6-11B-1 in addition to the metaphase band (Fig. 8, *e* and *f*).

The observation that the mutants contain more acetylated α-tubulin in the microtubules found in the cytoplasm than wild-type has been confirmed at the immunochemical level. Fig. 9 shows an immunoblot analysis of deflagellated cell body proteins from wild-type and the two *col*^{R} mutants separated on an SDS gel and probed with the two α-tubulin mAbs used in the immunofluorescence studies. While the total α-tubulin content of wild-type and mutant cells revealed by antibody 4.22.6 are similar, both mutant cell types possess a significantly higher proportion of acetylated α-tubulin than wild-type cells.

Discussion

In this study we have shown that the *col*^{R}*4* and *col*^{R}*15* β-tubulin mutations in *Chlamydomonas* confer a cross-resistance to the growth inhibitory effects not only of colchicine and vinblastine, two well-characterized microtubule-depolymerizing drugs, but also to three classes of chemically distinct antimitotic herbicides: the dinitroaniline herbicides oryzalin, trifluralin, profluralin, and ethafluralin, the phosphoric amide herbicide APM, and the dimethyl propynl benzamide herbicide, pronamide. These herbicides have been previously demonstrated to be potent microtubule poisons in a wide variety of higher plant cells and green algae; causing mitotic arrest at metaphase, the depolymerization of spindle and cytoplasmic microtubules, and the inhibition of flagellar assembly (for reviews see Fedtke, 1982; Morejohn and Fosket, 1986). In contrast to colchicine which is an effective microtubule inhibitor in algal and higher plant cells only at millimolar concentrations, the in vivo effects of these herbicides on microtubules are generally seen at submicromolar and low micromolar concentrations.

Although it has been controversial whether these herbicides directly interact with tubulin, recent studies have confirmed in the case of the dinitroanilines oryzalin and trifluralin that they bind to algal and plant tubulins with binding affinities similar to the binding of colchicine to animal tubulins ($K = 1–2 \times 10^5\ M^{-1}$) (Hess and Bayer, 1977; Strachan and Hess, 1983; Morejohn et al., 1987*b*). In addition, oryzalin and APM have been shown to inhibit the in vitro polymerization of higher plant tubulin and in the case of APM with no concomitant effect on the polymerization of brain tubulin (Morejohn and Fosket, 1984; Morejohn et al., 1987*a*). These data, together with the observation that the in vivo antimicrotubule effects are generally confined to nonanimal organisms suggest that these herbicides may interact with sites on plant and algal tubulins which may not be present in animal tubulins. Biochemical as well as amino acid sequence differences have been identified between plant and animal tubulins which may be correlated with functional differences such as herbicide binding (Silfow et al., 1987; Little et al., 1984; Morejohn and Fosket, 1986).

Chlamydomonas mutants isolated on the basis of resistance to the growth-inhibitory effects of the herbicides APM and oryzalin have been recently reported (James and Lefebvre, 1989; James et al., 1988, 1989). Although some of these mutations showed genetic interactions suggesting alterations in a common pathway, they were found to map to loci other than the α- or β-tubulin genes. In addition to these mutants, a dinitroaniline resistant biotype of the goosegrass *Eleusine* has been identified whose phenotype includes altered sensitivities to other microtubule inhibitors (Vaughn et al., 1987). A defect in the distribution of microtubules has been detected in the cytology of this resistant biotype, but the specific genetic lesion remains to be identified.

To our knowledge, *col*^{R}*4* and *col*^{R}*15* represent the first tubulin mutations identified in an algal or higher plant system. The observation that the mutations confer a cross-resistance to APM, the dinitroanilines, and pronamide provides the first genetic evidence that the in vivo antimitotic actions of these herbicides directly reflect their effects on microtubule function. This is significant because several of the herbicides have been reported to have, in addition to their antimicrotubule effects, other effects on cell functions (Hertel et al., 1980). In the case of APM and the various dinitroaniline herbicides, the level of resistance conferred by both the *col*^{R}*4* and *col*^{R}*15* mutations was similar to that observed for colchicine and vinblastine, approximately two- to threefold over wild type. Although colchicine and vinblastine bind to different sites on the tubulin dimer (Bryan, 1972), they have been shown to disrupt microtubules by a common mechanism involving the inhibition of assembly by substoichiometric binding to tubulin dimers rather than by the direct dissociation of formed microtubules (for a review see Wilson, 1975). Although there are no available data on the relationship of the tubulin-binding sites for APM and the dinitroaniline herbicides with each other or with the colchicine- and vinblastine-binding sites, the observation that the *col*^{R}*4* and *col*^{R}*15* mutations confer a similar level of resistance to each of these inhibitors suggests that their modes of action are similar. In comparison, the mutations conferred a 10-fold level of resistance to the pronamide, the active ingredient in

the commercial herbicide Kerb. Although we presently have no explanation for this enhanced resistance to pronamide, there is evidence which suggests that pronamide disrupts microtubules by a mechanism different from the dinitroanilines and APM. In contrast to other microtubule-depolymerizing drugs which cause a complete loss of plant spindle microtubules in vivo, onion root cells treated with pronamide and arrested at prometaphase have been found to contain an accumulation of short microtubules at the kinetochores of the chromosomes (Vaughan and Vaughn, 1987). The herbicide-resistant phenotypes of the *col^{R}4* and *col^{R}15* β-tubulin mutants suggest that, in addition to its well-documented usefulness as a model system for studying evolutionarily conserved features of the structure and function of microtubules (Huang, 1986; Lefebvre and Rosenbaum, 1986), *Chlamydomonas* represents an important genetically tractable system for the analysis of plant-specific properties of microtubules such as the mechanism of action of antimicrotubule herbicides.

The *col^{R}4* and *col^{R}15* mutations were also found to confer a super-sensitivity to the microtubule-stabilizing drug taxol paralleling the observations made by Cabral and his colleagues on the phenotypes of tubulin mutants in CHO cells in culture isolated on the basis of resistance to microtubule-depolymerizing drugs (Schibler and Cabral, 1985, 1986; Cabral et al., 1986). This pattern of altered sensitivities to different microtubule inhibitors is clearly not consistent with alteration in specific drug-binding sites. The simplest and most obvious explanation is that the *col^{R}4* and *col^{R}15* mutations increase the stability of the microtubules in the cells compared to wild type, and as a consequence, higher concentrations of microtubule-depolymerizing drugs and lower concentrations of the microtubule-stabilizing drug, taxol are needed to disrupt microtubule-dependent processes. Implicit in this model is that the mutant subunits are assembly competent. We have, in fact, demonstrated that the variant β-tubulins are assembled into the flagellar microtubules (Bolduc et al., 1988). Although it has not been documented at the biochemical level that the mutant tubulins are also assembled into the other microtubules found in *Chlamydomonas*, the observation that both mutations confer an increased resistance to the effects of colchicine on vegetative growth and cell division, as well as the germination of meiotic products during the sexual cycle (Bolduc et al., 1988) suggests that the variant tubulins are incorporated into the microtubules involved in these processes.

In the case of the *col^{R}4* and *col^{R}15* mutations, the extent to which the microtubules are stabilized by the mutations does not fall outside the range required for normal microtubule function. When grown in the absence of microtubule inhibitors the mutants have wild-type growth characteristics and have not been found to express conditional lethal phenotypes (Bolduc et al., 1988).

Direct evidence that the microtubules in vivo in the mutants have an enhanced stability was provided by our immunofluorescence analysis of the distribution of microtubules containing acetylated α-tubulin in the mutants as compared with wild type. It has been well documented that the α-tubulin subunit is subject to the posttranslational modification of lysine-ε-amino acetylation in the polymer phase (L'Hernault and Rosenbaum, 1983; LeDizet and Piperno, 1986). It has also been demonstrated that microtubules containing this posttranslational modification correspond to subsets of microtubules found in a variety of cells which turn over with distinctly slower kinetics than microtubules which are unmodified (Schulze et al., 1987; Piperno et al., 1987). As has been previously reported (LeDizet and Piperno, 1986; Holmes and Dutcher, 1989), we observed in wild-type *Chlamydomonas* cells that the flagellar microtubules and four cross-linked sets of flagellar microtubule roots in interphase cells, as well as a set of cytoplasmic microtubules found in mitotic cells (those comprising the metaphase band), contained acetylated α-tubulin; however, an extensive interphase array of cortical cytoplasmic microtubules and those comprising the intranuclear mitotic spindle were not acetylated. Like the unmodified microtubules found in mammalian cells in culture (Schulze et al., 1987), those found in wild-type *Chlamydomonas* cells are likely to be kinetically dynamic with half-lives too short to become acetylated. The observation that in both *col^{R}4* and *col^{R}15* cells these subsets of microtubules are acetylated indicates that these microtubules are turning over less rapidly than in wild type. This interpretation of the results is supported by our recent observation that wild-type *Chlamydomonas* cells grown in the presence of 5 μM taxol (which does not interfere with the growth characteristics of the cells) contain more acetylated microtubules, phenocopying the appearance of *col^{R}4* and *col^{R}15* mutants grown in the absence of any drugs (Schibler and Huang, unpublished results).

The data presented here underscore the importance of analyzing mutations which may have only subtle effects on microtubule structure and function. Definition of the precise nature of such mutations correlated with in vivo and in vitro analyses have the potential of providing more information on functionally important aspects of tubulin structure than lesions that may have catastrophic effects on microtubule assembly. In the case of the *col^{R}4* and *col^{R}15* β2-tubulin mutations, the mutant phenotypes have been found to be a consequence of substitutions of the amino acid residue lysine-350 with methionine or glutamic acid (Lee and Huang, 1990). Taken together, these data indicate that lysine-350 defines a functional site or domain in β-tubulin that is important for establishing the intrinsic stability of microtubules.

We thank Drs. Kevin Sullivan and Vincent Lee for their careful reading of the manuscript and helpful suggestions.

This work was supported by National Institutes of Health grant #GM-38113.

Received for publication 9 November 1990 and in revised form 28 January 1991.

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