Chlamydomonas α-Tubulin Is Posttranslationally Modified by Acetylation on the ε-Amino Group of a Lysine†

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ABSTRACT: Previous work has shown that the principal α-tubulin within *Chlamydomonas reinhardtii* flagellar axonemes differs from the major α-tubulin in the cell body. These two variants of α-tubulin are related to one another since posttranslational modification of the cell body form converts it to the axonemal form. When flagella are induced to assemble in the absence of de novo protein synthesis, tritiated acetate can be used to posttranslationally label α-tubulin in vivo, and under these conditions, no other flagellar polypeptides exhibit detectable labeling [L'Hernault, S. W., & Rosenbaum, J. L. (1983) *J. Cell Biol. 97*, 258–263]. In the present report, this labeling method has been used to provide material for chemical analysis of the tritiated moiety that is posttranslationally added to flagellar α-tubulin. This radioactivity was volatile after acid hydrolysis, suggesting that the posttranslational modification is the addition of neither an amino acid nor carbohydrate. Treatment of posttranslationally ^{3}H-labeled α-tubulin with hydrazine yields radioactive acetylhydrazine, indicating that the moiety involved in posttranslational modification is an acetyl group. Analysis of complete proteolytic digests by thin-layer chromatography has revealed that this acetyl group is located on the ε-amino group of a flagellar α-tubulin lysine residue.

Microtubules are ubiquitous constituents of eucaryotic cells and are major components of the mitotic spindle, cytoskeleton, cilia, and flagella [for a review, see Dustin (1978)]. The principal component of microtubules is the M_r 110000 protein tubulin (Mohri, 1968), which is a heterodimer composed of an α- and a β-polypeptide (Bryan & Wilson, 1971; Luduena et al., 1977). Mammalian brain is an especially rich source of tubulin, and many in vitro studies have defined the biochemical parameters that influence the nucleated self-assembly of brain tubulin into microtubules (Dustin, 1978; McKeithan & Rosenbaum, 1984; Scheele & Borisy, 1979). Recently, the complete amino acid sequences of brain α- and β-tubulin have been determined (Krauhs et al., 1981; Ponstingl et al., 1981).

The conserved nature of this protein was shown in several studies where α- and β-tubulin prepared from taxonomically distant species yielded highly similar peptide maps (Little et al., 1981) or had shared antigenic determinants (Brown et al., 1976; Dales, 1972; Fulton et al., 1971; Piperno & Luck, 1977). Tubulin or tubulin-containing organelles from diverse sources will respectively form copolymers or nucleate mammalian brain tubulin to assemble in vitro (Allen & Borisy, 1974; Binder et al., 1975; Burns & Starling, 1974; Kuriyama, 1976; Lai et al., 1979; Rosenbaum et al., 1975; Sheir-Neiss et al., 1976; Water & Kleinsmith, 1976; Weeks & Collis, 1976). While these studies demonstrate that tubulin is a highly conserved protein, microheterogeneities of α- and/or β-tubulins do exist, even within a single cell type (Fulton & Simpson, 1976; Gozes & Sweadner, 1981; Lefebvre et al., 1980; McKeithan & Rosenbaum, 1981; Sheir-Neiss et al., 1976; Stephens, 1977). Although several species contain multiple genes for α- and β-tubulin that could cause the microheterogeneities in these polypeptides (Brunke et al., 1982; Cleveland et al., 1980; Kalfayan et al., 1981; Silflow & Rosenbaum, 1981; Thomashow et al., 1983), in vivo posttranslational modification of tubulin has also been shown to occur. Previously described in vivo posttranslational modifications of tubulin include β-tubulin phosphorylation (Eipper, 1974) and removal of the mRNA-encoded carboxyl-terminal tyrosine from α-tubulin (Valenzuela et al., 1981) by a tubulin-specific carboxypeptidase (Kumar & Flavin, 1981). The reverse of this latter reaction, tyrosinolation, occurs by the addition of tyrosine to the penultimate carboxyl-terminal amino acid of α-tubulin (a glutamic acid residue) in a reaction that requires both ATP hydrolysis and the enzyme tubulin:tyrosine ligase (Arce et al., 1975; Argarana et al., 1977; Raybin & Flavin, 1975, 1977a,b). The functional significance of either β-tubulin phosphorylation or α-tubulin tyrosinolation/detyrosinolation is unknown.

A new type of tubulin posttranslational modification has recently been discovered in α-tubulin of the unicellular green alga *Chlamydomonas reinhardtii* (Lefebvre et al., 1980; L'Hernault & Rosenbaum, 1983; McKeithan et al., 1983). The major flagellar α-tubulin (α_3) of *Chlamydomonas* was shown to be synthesized as a precursor (α_1) that was posttranslationally modified within the flagella during their assembly (L'Hernault & Rosenbaum, 1983). The modifying moiety was removed during the flagellar disassembly that accompanies flagellar resorption.[1] The moiety involved in posttranslational modification of α-tubulin could be specifically labeled by incubating deflagellated cells in media containing both [^{3}H]acetate and the protein synthetic inhibitor cycloheximide. Although this result suggested α-tubulin acetylation, it was not conclusively proven since *Chlamydomonas* is an autotrophic organism that can utilize acetate as its carbon source. Consequently, direct chemical analysis of *Chlamydomonas* posttranslationally modified α-tubulin was performed in order to identify the modification. The results show that

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[1] See L'Hernault & Rosenbaum (1985).

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Chlamydomonas α-tubulin is posttranslationally modified by acetylation that occurs on the ε-amino group of lysine.

Experimental Procedures

Materials. L-Lysine, N^{α}-acetyl-L-lysine, N^{ϵ}-acetyl-L-lysine, and anhydrous hydrazine were purchased from Sigma (St. Louis, MO). Ninhydrin and constant boiling 6 N HCl were from Pierce (Rockford, IL) while L-1-(tosylamido)-2-phenylethyl chloromethyl ketone (TPCK) treated trypsin and α-chymotrypsin were from Millipore (Freehold, NJ). Pronase and carboxypeptidase Y were from Calbiochem (La Jolla, CA). Silica gel F (250-μm thick-layer) and cellulose (100-μm thick-layer without fluorescence indicator) chromatography plates on plastic backing were from EM Laboratories (Elmsford, NY). Aquasol was from New England Nuclear (Boston, MA) while Autofluor was from National Diagnostics (Somerville, NJ). Solvents utilized in thin-layer chromatography were reagent grade or better while all other materials were as described previously (L'Hernault & Rosenbaum, 1983).

In Vivo Labeling. Cells were labeled in vivo with [^{3}H]-acetate according to procedures that have been discussed previously (L'Hernault & Rosenbaum, 1983) and will be briefly outlined. The objective of these labeling experiments was to discover a method of identifying the posttranslational modification on α_3-tubulin by radiochemical labeling. In vivo, this posttranslational modification occurs within flagella during flagellar assembly and converts the major cytoplasmic α-tubulin (α_1) into the major flagellar axonemal α-tubulin (α_3).

Normally, deflagellation of *Chlamydomonas* induces the synthesis of many flagellar proteins simultaneously with the regeneration of new flagella (Lefebvre et al., 1978). *Chlamydomonas*, which is an autotroph, can synthesize amino acids from simple compounds such as acetate or sulfate and utilize them to label many (if not all) flagellar proteins during translation (Rosenbaum et al., 1969; Lefebvre et al., 1978). When de novo protein synthesis is inhibited by cycloheximide, *Chlamydomonas* utilizes a cytoplasmic precursor pool as its exclusive source of flagellar protein and is capable of assembling two half-length flagella (Rosenbaum et al., 1969; Lefebvre et al., 1978). When [^{3}H]acetate is added to cells that are assembling flagella in the presence of cycloheximide, analysis of the resulting flagellar axonemes on two-dimensional (2-D) gels reveals that only α_3-tubulin exhibits detectable ^{3}H labeling (Figure 1). These analytical 2-D gels provided the purified posttranslationally labeled α_3-tubulin used in the present study. Although we were only able to obtain limited quantities of material that had been posttranslationally ^{3}H labeled in vivo, the previously demonstrated specificity of this labeling (L'Hernault & Rosenbaum, 1983) permitted the qualitative analyses reported below.

Processing of 2-D Gels. Regions containing posttranslationally labeled α_3-tubulin were excised from fixed 2-D gels. This polyacrylamide plug was rinsed 3 times with distilled water (5 min per change) to remove the water-soluble Autofluor and then rinsed briefly with 100 mM NH_4HCO_3 to neutralize remaining traces of acetic acid. The gel was then macerated in 500 μL of 100 mM NH_4HCO_3, and the radioactive α_3-tubulin was solubilized by proteolysis. The resulting radioactive α_3 proteolytic products, unlike the parent polypeptide, were soluble in aqueous solutions in the absence of chaotropic agents. Proteolytic digestion was performed with trypsin (20 μg added as two 10-μg aliquots at 12-h intervals; 37 °C) or by the successive addition of Pronase, α-chymotrypsin, and carboxypeptidase Y (Granger et al., 1976; Woodford & Dixon, 1979). Following the completion of proteolytic digestion, macerated gel fragments were then pelleted by centrifugation at 12000*g* for 1 min in an Eppendorf centrifuge (Model 5414, 15 000 rpm, Brinkman Instruments, Westbury, NY). The supernatant was saved, and the gel pieces were rinsed with three 250-μL aliquots of 100 mM NH_4HCO_3 and repelleted at 12000*g* (as above) between each 5-min rinse. The rinses were pooled with the initial supernatant and lyophilized in a Speed-Vac centrifuge (Savant Instruments Inc., Hicksville, NY). The lyophilized peptides were resuspended in 100 μL of 50 mM NH_4HCO_3, sonicated for 2 min (Model B-12, Branson Cleaning Equipment Co., Shelton, CT), and subjected to centrifugation at 12000*g* for 10 min in an Eppendorf centrifuge (as above) to pellet insoluble material. Duplicate aliquots were added to 10-mL volumes of Aquasol, and the incorporated radioactivity was determined by liquid scintillation counting. The final supernatant of tryptic peptides in 50 mM NH_4HCO_3 served as the starting material for acid hydrolysis and hydrazinolysis experiments. Thin-layer chromatography was performed on the amino acids prepared by successive digestion of posttranslationally ^{3}H-labeled α_3-tubulin with Pronase, α-chymotrypsin, and carboxypeptidase. These proteolytic methods solubilized greater than 85% of the ^{3}H-labeled α_3-tubulin in polyacrylamide gel pieces and provided a total of $\sim 1.3 \times 10^5$ cpm of labeled peptides for the chemical analyses described below.

Acid Hydrolysis. Acid hydrolysis was performed by adding 250 μL of 6 N HCl directly to 20-μL aliquots of tryptic digest and permitting hydrolysis to occur for 18 h at 105 °C. The resulting hydrolysate was transferred to a microchemical distillation apparatus that allowed collection of volatile and nonvolatile fractions. These fractions were added to Aquasol for liquid scintillation counting.

Hydrazinolysis. Aliquots of tryptic peptide-containing solution were lyophilized in ampules and resuspended in 100 μL of anhydrous hydrazine. Hydrazinolysis was permitted to occur for 24 h at 80 °C (Narita, 1970, 1972). Hydrazine was removed under reduced pressure, the resulting hydrazinolysis products were dissolved in 20 μL of distilled water, and duplicate aliquots were added to 10-mL volumes of Aquasol for liquid scintillation counting.

Thin-Layer Chromatography. Aliquots (2 μL) of the hydrazinolysis products were spotted on cellulose thin-layer plates in parallel with 50 nM aliquots of formyl-, acetyl-, and propionylhydrazine standards [synthesized as described; Narita (1970)] and subjected to ascending chromatography in either pyridine/aniline/water (9:1:4) or collidine/water (5:1). Following chromatography, the lane containing the hydrazinolysis products of the α_3-tubulin peptides was cut off the chromatograph, and the rest of the plate (containing the standards) was stained with silver as described (Narita, 1970). This indirect method had to be employed because it was not possible to determine incorporated radioactivity in thin-layer chromatographs that had been silver stained. The portion of the plate containing the tubulin chromatographic products was cut into 1-cm strips. These strips were placed into vials, 100 μL of water was added, and the capped vials were heated at 60 °C for 1 h. The cellulose layer was scraped from its backing (but left in the vial), and 10 mL of Aquasol was added prior to liquid scintillation counting. Liquid scintillation counting times were chosen so that the final counting error of chromatogram regions containing the radioactivity peak did not exceed 2%.

The complete proteolytic digest was chromatographed in parallel with 1 μM each of L-lysine, N^{α}-acetyl-L-lysine, and N^{ϵ}-acetyl-L-lysine (1 μL of 1 M stock solutions) on silica gel

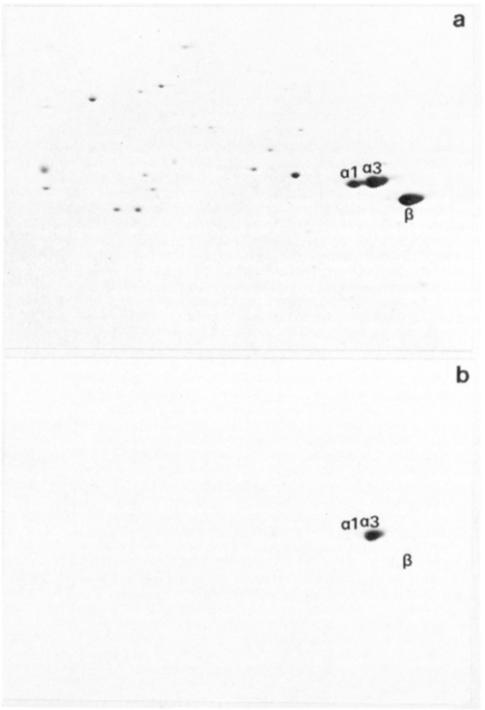

FIGURE 1: Posttranslational labeling of *Chlamydomonas* flagellar axonemes. Deflagellated cells were labeled in vivo with [^{3}H]acetate and permitted to regenerate flagella while de novo protein synthesis was inhibited. These flagella were amputated, and their axonemes (35 μg; 2 × 10^4 cpm) were subjected to two-dimensional electrophoresis (for details see Experimental Procedures). The same gel was first Coomassie blue stained (a) and then, subsequently, fluorographed for 24 h (b). Although many axonemal proteins, including α_1-, α_3-, and β-tubulin, are visible in the stained gel (a), α_3-tubulin is the only polypeptide that is detectably labeled under these conditions.

plates. Amino acid standards were chromatographed together with the proteolytic digestion products of nonradioactive α_3-tubulin. This nonradioactive tubulin was contained within a 2-D gel polyacrylamide plug that was prepared and processed in the same manner as radioactive α_3-tubulin (see above). Ascending chromatography was performed in either 1-butanol/acetic acid/water (4:1:1), 2-propanol/34% NH_4OH (7:3), ethanol/34% NH_4OH/water (7:1:2), or 1-butanol/benzyl alcohol [1:1 mixture that was saturated with water before use; Pataki (1968)]. Following chromatography, the lane containing the proteolytic digest of α_3-tubulin was cut off of the chromatogram prior to visualizing the standards with 0.5% ninhydrin (w/v) in acetone containing 1% pyridine (v/v). The chromatogram lane containing the proteolytic digest was cut into 1-cm strips, placed in liquid scintillation counting vials, and hydrated with 500 μL of water. The silica was then scraped from its plastic backing (but not removed from the vial), and 10 mL of Aquasol was added to each vial. Occasionally, standards were cochromatographed with the complete proteolytic digest of radioactive α_3-tubulin, and the resulting ninhydrin-stained standard spot was cut out and processed for liquid scintillation counting (as for the 1-cm strips). Liquid scintillation times were chosen so that the final counting error of chromatogram regions containing the radioactivity peak did not exceed ±2%.

Results

Previous results have shown that *Chlamydomonas* α-tubulin is reversibly posttranslationally modified in a flagellar assembly-dependent fashion (Lefebvre et al., 1980; L'Hernault & Rosenbaum, 1984; McKeithan et al., 1983).[1] It was discovered that radioactivity (derived from [^{3}H]acetate) could be found

Table I: Vacuum Distillation of Acid Hydrolysate of Posttranslationally ^{3}H-Labeled α_3-Tubulin[a]

	cpm hydrolyzed	nonvolatile cpm	volatile cpm
expt 1	2810	167 (10%)	1439 (90%)
expt 2	2360	148 (10%)	1339 (90%)
	2360	139 (9%)	1361 (91%)

[a] Vacuum distillation was performed in a microchemical distillation apparatus that allowed the collection of volatile and nonvolatile fractions. The results from two separate preparations (experiments 1 and 2) of posttranslationally labeled α_3-tubulin are presented; two aliquots of the preparation used in experiment 2 were separately hydrolyzed to illustrate the reproducibility of the technique. Both volatile and nonvolatile fractions were subjected to liquid scintillation counting in the presence of equal quantities of HCl.

on flagellar α-tubulin (α_3) in vivo in experiments where de novo *Chlamydomonas* protein synthesis was inhibited (Figure 1). Although these data suggested that α-tubulin might be acetylated, they did not provide conclusive proof since simple compounds such as acetate can be used as the sole carbon source of an autotrophic organism such as *Chlamydomonas*. Among carbon-containing moieties that have been previously shown to be involved in other posttranslational modifications, many could be ruled out. For instance, since the isoelectric point (p*I*) of α_3-tubulin (the posttranslationally modified form) is lower than that of α_1-tubulin [the precursor form; Lefebvre et al. (1980)], methylations (which raise p*I*'s) cannot be involved. ADP ribosylations, which would lower the p*I*, were ruled out by showing that ^{32}P could not be used to label α-tubulin in vivo (data not shown). Tyrosinolation, which is a common α-tubulin posttranslational modification in vertebrates (Raybin & Flavin, 1977), does not alter the p*I* of the modified polypeptide. In fact, acetylation was the most likely candidate for the *Chlamydomonas* α-tubulin posttranslational modification, and experiments were designed to test this hypothesis.

Acetylations are known to occur on proteins at free amino groups including both the amino terminus and the ε-amino group of lysine [reviewed in Wold (1981)]. In both cases, the acetyl group is part of an amide and, following acid hydrolysis, should yield acetic acid. To test this idea, α_3-tubulin that had been posttranslationally ^{3}H labeled (L'Hernault & Rosenbaum, 1983) was subjected to acid hydrolysis (see Experimental Procedures), and the resulting acid hydrolysate was vacuum distilled in a microchemical distillation apparatus that allowed collection of volatile and nonvolatile fractions. Liquid scintillation counting of these two fractions revealed that the bulk (90%) of the recovered radioactivity that had been posttranslationally added to α_3-tubulin was volatile after 6 N HCl hydrolysis (Table I).

Protein acetylations can be identified by first treating the protein with hydrazine and then analyzing the reaction products for the presence of acetylhydrazine by thin-layer chromatography (Narita, 1970, 1972). Therefore, posttranslationally ^{3}H-labeled α_3-tubulin tryptic peptides (see Experimental Procedures) were subjected to hydrazinolysis, and the resulting products were compared to synthesized standards by thin-layer chromatography in two solvent systems (Figure 2). In both cases, the major peak of radioactivity comigrated with the acetylhydrazine standard. These results provide strong evidence that *Chlamydomonas* α-tubulin is posttranslationally modified by acetylation.

In vivo protein acetylations occur on both the amino (N) terminus and the ε-amino group of internal lysine residues. In the case of histones, which are among the most extensively studied acetylated proteins [reviewed by Allfrey (1977), Dixon et al. (1975), Doenecke & Gallwitz (1982), and Isenberg

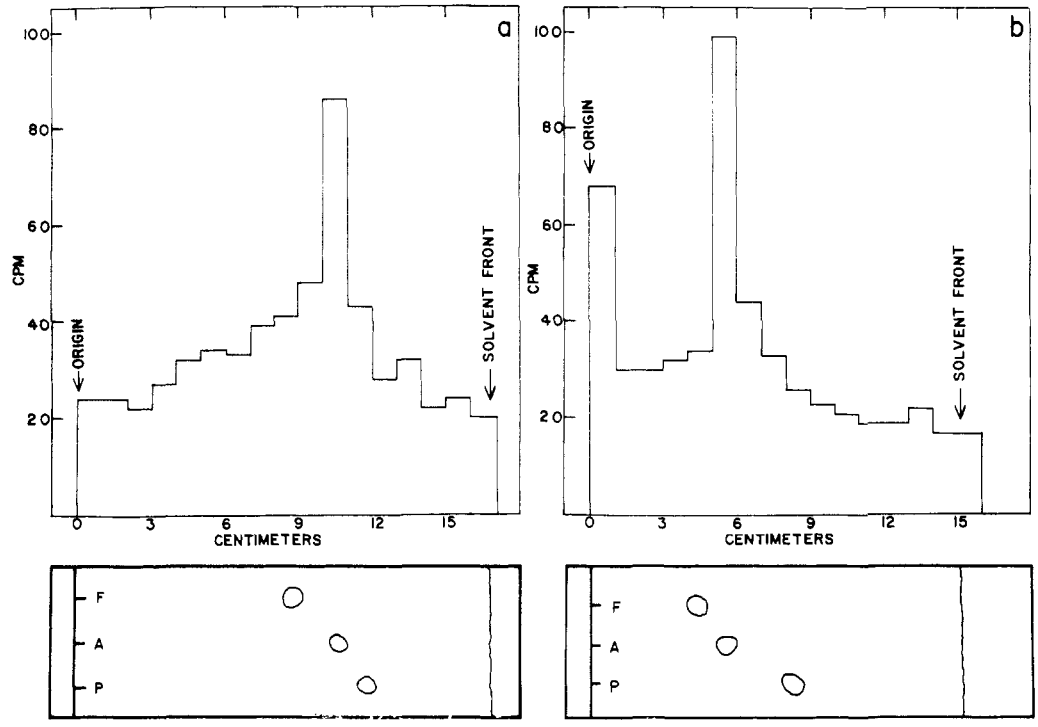

FIGURE 2: Thin-layer chromatography of hydrazinolysis products of posttranslationally ^{3}H-labeled α_3-tubulin. Hydrazinolysis products of α_3-tubulin were chromatographed in parallel with the standards formylhydrazine (F), acetylhydrazine (A), and propionylhydrazine (P). The two solvent systems employed were (a) pyridine/aniline/water (9:1:4) and (b) collidine/water (5:1). Beneath the histogram, which represents the distribution of α_3-tubulin-derived ^{3}H, are tracings of the standards that were chromatographed in parallel and stained with silver. Chromatograms were loaded with 300 cpm of ^{3}H-labeled α_3-tubulin hydrazides.

(1979)], N-terminal acetylations are believed to be irreversible and occur while the nascent polypeptide is still being synthesized (cotranslational modification). Acetylations of the ϵ-amino groups on internal lysine residues, however, are reversible, do not require ongoing protein synthesis, and can occur within an intracellular compartment, the nucleus. Since the characteristics of *Chlamydomonas* α-tubulin acetylation are remarkably similar to histone lysine ϵ-amino group acetylation [except, in the case of *Chlamydomonas* α-tubulin, the intracellular compartment is the flagellum; L'Hernault & Rosenbaum (1983)], we performed thin-layer chromatography on complete proteolytic digests of posttranslationally ^{3}H-labeled α_3-tubulin to search for this modified amino acid. Chromatography was performed in four solvent systems of differing hydrophobicity and pH, and in each case, the peak of radioactivity in the α_3-tubulin digest comigrated with authentic acetylated lysines (Figure 3). Chromatography of α_3-tubulin digests in the ammonia-containing solvents, with their excellent resolution of N^{α}-acetyllysine from N^{ϵ}-acetyllysine, indicates that the [^{3}H]acetyl group is located on an ϵ-amino group of a lysine residue (Figure 3c,d). Since a pronounced and reproducible chromatographic artifact (presumably due to contaminating salt) was experienced in one solvent system (Figure 3c), we cochromatographed the α_3-tubulin digest together with N^{ϵ}-acetyllysine, ninhydrin stained the chromatogram, and excised both the stained spot and the (unstained) surrounding regions, including the trough of the heart-shaped spot (as in Figure 3c). In all cases, the only significant radioactivity was found within the ninhydrin-stained N^{ϵ}-acetyllysine spot (data not shown). These data indicate that *Chlamydomonas* α_3-tubulin is acetylated on the ϵ-amino group of a lysine residue(s).

Discussion

The posttranslational modification (acetylation) of *Chlamydomonas* α_3-tubulin previously has been shown to be coupled to flagellar assembly (L'Hernault & Rosenbaum, 1983). Although the involved acetylase has not yet been isolated, these studies also suggested that the presumptive acetylating enzyme was activated upon its transport into the growing flagellum. Apparently, acetylation occurs at or just before the time of tubulin dimer subunit addition to the growing axonemal microtubules (L'Hernault & Rosenbaum, 1983). These acetylated axonemal microtubules, apparently, are deacetylated as they are nonproteolytically disassembled during flagellar resorption.[1] This reversibility suggests that α-tubulin acetylation has a function in the assembly and disassembly of *Chlamydomonas* axonemes.

The results also indicate that *Chlamydomonas* α_3-tubulin is reversibly acetylated on the ϵ-amino group of a lysine residue(s). Acetylations at this site are observed far less frequently than the commonly occurring N-terminal acetylations [reviewed by Wold (1981)] and, to date, have been found only in histones and several nonhistone nucleosomal "high mobility group" proteins (Sterner et al., 1978, 1979, 1982, 1983). Due to their reversible nature, acetylations of internal histone lysine residues are believed to modulate the binding of these proteins to DNA [reviewed by Allfrey (1977), Dixon et al. (1975), Doenecke & Gallwitz (1982), and Isenberg (1979)]. Although determining the precise function of *Chlamydomonas* α_3-tubulin acetylation will require further study, there are several possible roles that this posttranslational modification might play.

The most obvious potential role for *Chlamydomonas* tubulin acetylation would be to impart structural stability to the flagellar axoneme. The biophysical characteristics of flagellar microtubules are, generally speaking, quite different from cytoplasmic microtubules; the former are cold, pressure, and colchicine insensitive while the latter are disrupted by any of these treatments (Behnke & Forer, 1967). Acetylation may permit tubulin to interact with other proteins to form the outer doublet structure, which is a characteristic of cilia and flagella

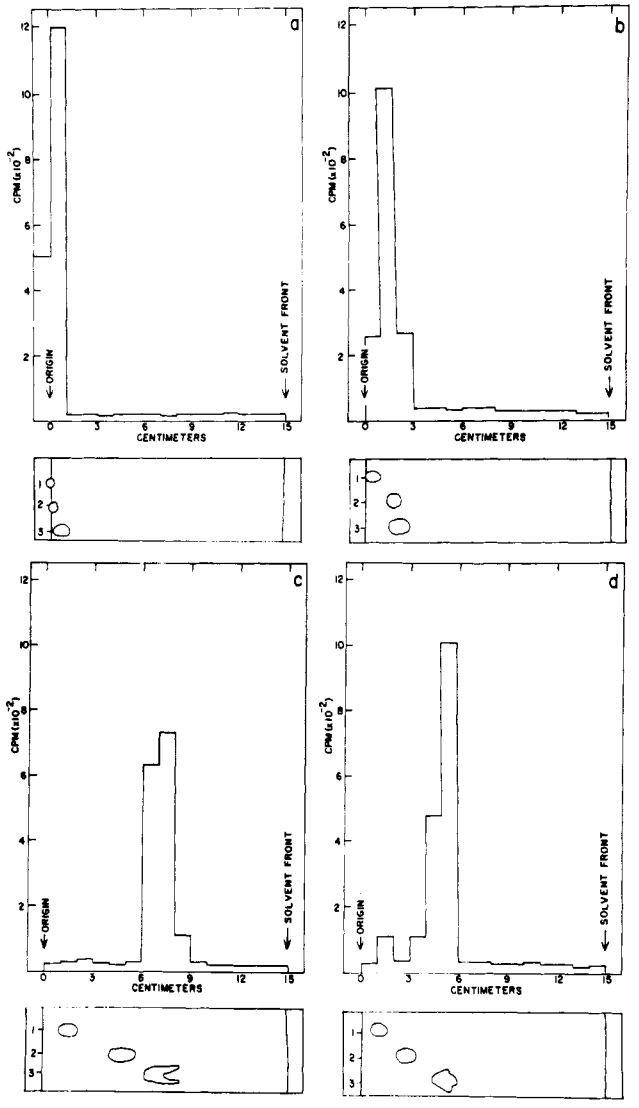

FIGURE 3: Thin-layer chromatography of complete proteolytic digest of posttranslationally ^{3}H-labeled α_3-tubulin. Proteolytic products of α_3-tubulin were chromatographed in parallel with the standards L-lysine (1), N^{α}-acetyl-L-lysine (2), and N^{ϵ}-acetyl-L-lysine (3). The four solvent systems employed were (a) 1-butanol/benzyl alcohol (1:1 mixture that was saturated with water before use), (b) 1-butanol/glacial acetic acid/water (4:1:1), (c) ethanol/34% NH_4OH/water (7:1:2), and (d) 2-propanol/34% NH_4OH (7:3). Beneath the histogram, which represents the distribution of α_3-tubulin-derived ^{3}H, are tracings of standards that were chromatographed in parallel and stained with ninhydrin. All chromatograms were loaded with 2000 cpm of ^{3}H-labeled α_3-tubulin proteolytic products.

but not of cytoplasmic microtubules [reviewed in Dustin (1978)].[2] Alternatively, binding of accessory structures (e.g., nexin links, dynein, radial spokes) may be facilitated by tubulin acetylation. Appropriate inhibitor experiments, perhaps with the competitive histone acetylation donor 2-mercaptoacetate (Sterner & Allfrey, 1982, 1983), may perturb the normal function of tubulin acetylation and thus identify its role. Additional useful and complementary data might be obtained if one could inhibit the apparent deacetylation that occurs during the axonemal disassembly that accompanies flagellar resorption.[1] This might be possible if the histone deacetylase inhibitor butyrate (Riggs et al., 1977) was effective in *Chlamydomonas*.

[2] Flagellar fractionation (our unpublished observations) and the analysis of mutants (Adams et al., 1981) have both shown that removal of the central pair microtubules does not remove all of the α_1- (precursor) tubulin from the *Chlamydomonas* flagellar axoneme. The distribution of posttranslationally modified α-tubulin (α_3) within outer doublet A or B subfibers has not yet been determined.

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Biosynthesis of Fredericamycin A, a New Antitumor Antibiotic†

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ABSTRACT: Fredericamycin A (FM A), produced by a strain of *Streptomyces griseus*, represents a new structural class of antitumor antibiotics containing a spiro ring system. Studies on the producer organism showed that glucose in the fermentation medium is not utilized until late in the growth stage, just prior to synthesis of FM A. [^{14}C]Glucose tracer experiments demonstrated that glucose is incorporated into FM A by catabolism to acetate. Biosynthetic enrichment of FM A with single- and double-labeled [^{13}C]acetate showed that the entire carbon skeleton of the spiro ring system is derived from acetate. L-Methionine was shown to provide the only nonskeletal carbon in FM A, the methoxy carbon at position C-6. The direction of the polyketide chain and the position of the carbon lost during biosynthesis were established by using stable isotope experiments. A general model for FM A biosynthesis is proposed, and a possible scheme for the formation of the spiro carbon center is presented.

Fredericamycin A (FM A)[1] (NSC 305263) is an antitumor antibiotic produced by *Streptomyces griseus* (FCRC-48) (Pandey et al., 1981). In addition to possessing in vitro activity against Gram-positive bacteria and fungi, FM A was shown

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[1] Abbreviations: FM A, fredericamycin A; Me_2SO, dimethyl sulfoxide; TFA, trifluoroacetic acid; CoA, coenzyme A; NMR, nuclear magnetic resonance; HPLC, high-performance liquid chromatography; Me_4Si, tetramethylsilane.

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