

Reversal of the Posttranslational Modification on *Chlamydomonas* Flagellar α -Tubulin Occurs during Flagellar Resorption

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ABSTRACT We previously have shown that a posttranslational modification of α -tubulin takes place in the flagellum during *Chlamydomonas* flagellar assembly (L'Hernault, S. W., and J. L. Rosenbaum, 1983, *J. Cell Biol.*, 97:258–263). In this report, we show that the posttranslationally modified α -3 tubulin is changed back to its unmodified α -1 precursor form during the microtubular disassembly that takes place during flagellar resorption. These data indicate that the addition and removal of a posttranslational modification on α -tubulin might be a control step in the assembly and disassembly of flagella.

Recently, it has been discovered that the major *Chlamydomonas* flagellar α -tubulin, α -3, is a posttranslationally modified form of α -1, the major α -tubulin residing in the cell body, and that this modification is coupled to flagellar assembly (5, 18–22, 26). If this modification is a control step in flagellar assembly, then one might expect its reversal during flagellar disassembly. Flagellar disassembly accompanies flagellar resorption in *Chlamydomonas*, and previous results suggest that the proteins from disassembled flagella appear in the cell body during this process (17, 19). As this should permit the study of α -tubulin derived from disassembled flagella, we sought to ascertain if α -tubulin posttranslational modification was reversible during flagellar resorption. We found that the posttranslationally added moiety on flagellar α -tubulin (α -3) appears to be specifically removed during flagellar resorption. An initial report of these results has been presented (20).

MATERIALS AND METHODS

Materials: $^{35}\text{SO}_4$ (as $\text{H}_2^{35}\text{SO}_4$, carrier free, 43 Ci/mg), Liquifluor[®] and Protosol[®] were purchased from New England Nuclear (Boston, MA) while all other materials were as described previously (21).

Cell Culture and In Vivo Labeling: Wild-type *Chlamydomonas reinhardtii*, strain 21gr vegetative cells were used in all experiments. Conditions of growth in low sulfur minimal medium 1, mechanical shear deflagellation, flagellar length determination and in vivo labeling were similar to those previously described (17) with the modifications described below.

Deflagellated cells, which had been ^{35}S -pulse-chase-labeled during a flagellar regeneration before the induction of flagellar resorption, were used in all experiments. To perform this type of labeling, we deflagellated cells grown in

low sulfur minimal medium (17) and labeled them with 400 $\mu\text{Ci/ml}$ ^{35}S for the first 10 min following deflagellation. Cells were pelleted by centrifugation at 460 g (IEC, PR-6 model centrifuge, 269 rotor, 1,600 rpm, Damon/IEC, Needham Heights, MA), washed, repelleted, and resuspended in a modified medium 1 (35). This modified medium 1 lacked KH_2PO_4 , contained 5 mM Na_2SO_4 as a chase, and had a pH of 7.5 (high pH chase medium). After 80 min of flagellar regeneration under chase conditions, cycloheximide (10 $\mu\text{g/ml}$) was added and the cells were divided into two equal aliquots. The aliquot under control conditions received HEPES buffer (pH 7.5 with NaOH, final medium concentration 15 mM HEPES), while flagellar resorption was induced in the cells of the other aliquot by the addition of sodium pyrophosphate (pH 7.5 with HCl, final medium concentration, 15 mM sodium pyrophosphate). A pH of 7.5, rather than the previously reported pH 6.8 (17), was used in these experiments because it was found that flagellar resorption was more uniform at this pH. HEPES was chosen as the control buffer because, unlike phosphate buffers, it did not cause detectable flagellar shortening. Under these conditions, pyrophosphate induces complete flagellar resorption in ~ 90 min, while the cells under control conditions (HEPES buffer addition) maintain full-length flagella. After completion of flagellar resorption, aliquots of cells under both control and resorption inducing conditions were pelleted by centrifugation (460 g , as above), washed once with high pH chase medium containing cycloheximide (10 $\mu\text{g/ml}$), and repelleted.

Sample Preparation and Two-dimensional Electrophoresis: Cells were deflagellated with dibucaine and fractionated into cell bodies and flagella as described previously (21, 44, 46), except in in vivo labeling experiments in which flagella were pelleted by centrifugation at 146,000 g (Spinco model L2-65b ultracentrifuge, 50 Ti type rotor, 47,000 rpm, Beckman Instruments Inc., Palo Alto, CA) for 30 min to ensure that even short flagella would pellet (30). Electrophoretic comparisons of total cellular protein were performed on two-dimensional (2-D)¹ gels that had been loaded with an equal

¹Abbreviations used in this paper: 2-D, two-dimensional; TCA, trichloroacetic acid.

number of counts per minute of radioactive protein. Cell counts were taken just before 2-D gel sample preparation. Equivalent numbers of cells were found to contain the same amount of radioactivity (data not shown). Cellular fractionations were performed on equal numbers of cells and the resulting cell bodies and flagella were compared by electrophoresis. Cells, cell bodies, and flagella were prepared for 2-D electrophoresis, electrophoresed, stained (21), and autoradiographed (17) as described previously.

Gel Quantitation: All gels were co-electrophoresed with 25 μg of nonradioactive *Chlamydomonas* flagellar axonemes (prepared as described [21]), fixed, stained, and autoradiographed (as above). Radioactive tubulin regions of 2-D gels were located by co-migration with Coomassie Blue-stained nonradioactive flagellar protein standards and alignment with autoradiographs. In the case of α -3 tubulin, the major 2-D gel spot commonly had a smaller spot closely apposed to its acidic side. This closely apposed spot is artifactually modified from the polypeptide that is in the major spot (data not shown), so each major and modified spot were treated as a single entity. Radioactive gel regions were excised from dried gels with a scalpel, placed in a scintillation counting vial, and rehydrated with 100 μl of water. The reswollen gel was then digested with 0.5 ml of Protosol[®] in a tightly sealed vial at 55°C for 24 h. Samples were cooled to room temperature, 10 ml of Liquifluor[®] was added, and liquid scintillation counting was performed.

RESULTS

The purpose of the present study was to monitor changes in tubulin and other proteins derived from flagella as they appeared in the cell body during flagellar resorption. The main difficulty with this type of analysis is that *Chlamydomonas* flagellar proteins exist not only assembled within flagella but also in an unassembled cytoplasmic precursor pool (17, 31). Consequently, after the completion of flagellar resorption, the cell body contains flagellar proteins derived from both disassembled flagella and the flagellar precursor pool. To distinguish between these two groups of proteins, we prepared cells that had ³⁵S-labeled flagella with minimal labeling of the flagellar precursor pool proteins. This was achieved by pulse-chase labeling deflagellated cells during a flagellar regeneration that preceded the induction of flagellar resorption. The characteristics of this labeling method will be described before presentation of the evidence that it differentially labels flagella.

Kinetics of Pulse-chase Labeling during Flagellar Regeneration

Incorporation of label into trichloroacetic acid (TCA)-precipitable protein began when cells were placed in ³⁵S-containing medium shortly after deflagellation and continued to occur in a linear fashion for at least 90 min (Fig. 1, open circles). If pulse-labeled cells are washed out of ³⁵S-containing media after 10 min of labeling (Fig. 1, arrow at P) and placed under chase conditions (see Materials and Methods), no significant additional ³⁵S incorporation into TCA-precipitable protein occurs (Fig. 1, solid squares). These pulse-chase-labeled cells have regenerated full-length flagella by 90 min (arrow at C, Fig. 1) and were subsequently used in flagellar resorption experiments. Even though net incorporation of ³⁵S into TCA-precipitable protein was prevented during the chase (Fig. 1, solid squares), flagellar resorption experiments were performed in the presence of cycloheximide (added at arrow at C, Fig. 1) to ensure that labeling did not occur from ³⁵S turnover during the required time interval (Fig. 1, solid circles). A quantity of cells equal to that in which resorption was induced served as a control (Fig. 1, open squares). Medium in which control cells were placed was of similar ionic strength, identical pH, and contained cycloheximide, but did not induce flagellar resorption. Comparison of this control (Fig. 1, open squares) to cells that were resorbing their flagella

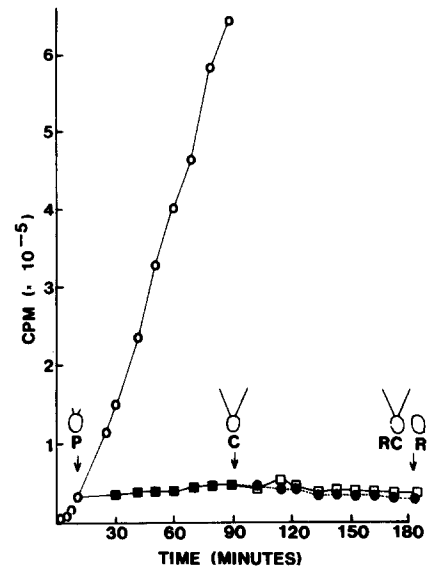


FIGURE 1 Incorporation of ³⁵S into *Chlamydomonas* TCA-precipitable protein. Deflagellated cells were pulse-labeled with ³⁵S (open circles); after 10 min of labeling, most of the cells were pelleted by centrifugation, resuspended under chase conditions (solid squares), and allowed to incubate for an additional 80 min (see Materials and Methods). Cycloheximide (C) was then added to these pulse-chase-labeled cells and they were divided into two aliquots that were either induced to resorb their flagella (R) (dashed line connecting solid circles) or placed under nonresorbing control conditions (RC) (solid line connecting open squares). Means of two determinations are plotted and the differences between resorb and resorb control samples are not significant. Cartoons depict flagellar lengths on cells at points indicated by the arrows. Arrows also indicate times at which aliquots were prepared for 2-D electrophoresis. P, end of pulse-labeling and the beginning of chase conditions; C, addition of cycloheximide to pulse-chase-labeled cells; R and RC, end of the time interval required for flagellar resorption (R) and its control (RC).

(Fig. 1, solid circles) reveals that the net levels of TCA-precipitable ³⁵S-labeled proteins are similar under these two conditions.

Monitoring of Flagellar Length during In Vivo Labeling

Flagellar regeneration during pulse-chase labeling follows the deceleratory kinetics (Fig. 2, open circles) that have been described previously (17, 31). At 90 min, when flagella were nearly full-length, cells were either placed under flagellar resorption conditions (Fig. 2, solid circles) or control conditions (Fig. 2, open squares). Cells under resorption-inducing conditions rapidly shortened their flagella so that they were flagellaless (by phase-contrast optics) after 90 min (Fig. 2, solid circles). No significant change in flagellar length occurs in cells maintained under control conditions for 90 min (Fig. 2, open squares).

Electrophoretic Analysis

Samples were prepared for electrophoretic analysis after 90 and 180 min, as indicated in Fig. 1. Cells that had completed pulse-chase labeling (arrow under C, Fig. 1) and cells that had resorbed their flagella or were a control for this condition (arrows under RC and R, Fig. 1) were analyzed and autora-

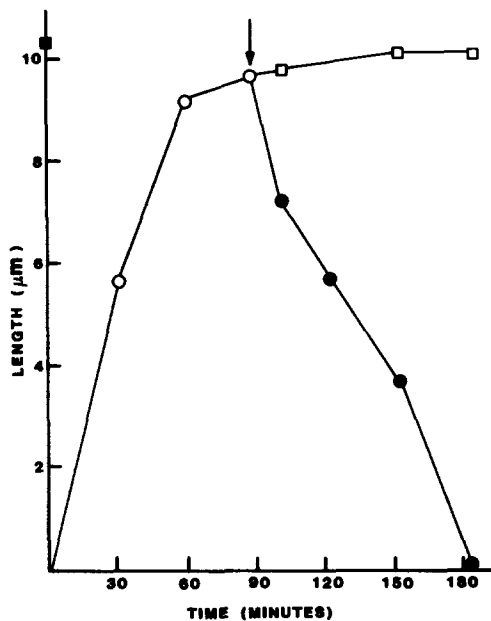


FIGURE 2 Flagellar regeneration and resorption kinetics. Deflagellated cells were permitted to regenerate flagella (open circles) during pulse-chase ^{35}S -labeling (as in Fig. 1). Cycloheximide was added at the indicated time (arrow) when aliquots of cells were either placed under control (open squares) or flagellar resorption (solid circles) conditions. The solid square on the ordinate indicates the flagellar length before deflagellation.

diographs of the resulting 2-D gels appear in Fig. 3. We have analyzed the entire cell (Fig. 3, *a*, *d*, and *g*) and cells fractionated into cell bodies (Fig. 3, *b*, *e*, and *h*) and flagella (Fig. 3, *c*, *f*, and *i*). While the principal purpose of this study was the analysis of tubulin, we have also studied the distribution of three other proteins (Fig. 3, arrows and arrowheads) that illustrate how proteins can be compartmentalized and/or metabolized during flagellar resorption.

2-D electrophoretic analysis of whole cells that have been ^{35}S -pulse-chase labeled during flagellar regeneration reveals that they contain substantial labeled tubulin (Fig. 3*a*). The principal α -tubulin observed under these conditions was α -3, which has previously been shown to be the principal flagellar α -tubulin (18–22, 26). When pulse-chase labeled cells (as in Fig. 3*a*) were fractionated into cell bodies (Fig. 3*b*) and flagella (Fig. 3*c*), most of the tubulin partitions with the flagella. A further indication of the specificity of flagellar labeling is obtained by analyzing another flagellar protein. This flagellar protein (arrows, Fig. 3, *a*, *b*, and *c*), unlike tubulin, has insignificant cytoplasmic precursor pool labeling under these conditions (22) and the labeled polypeptide partitions specifically with flagella following cell fractionation (compare Fig. 3, *b* and *c*). These results indicate that our labeling conditions differentially label flagellar proteins but, in the case of tubulin, some cytoplasmic precursor labeling is unavoidable. This level of precursor labeling was sufficiently low to permit the analysis of labeled proteins derived from resorbed flagella after their appearance in the cell body (see below).

Control cells, which have nonresorbing full-length flagella (Fig. 3*d*), have a 2-D gel autoradiographic pattern that is highly similar to pulse-chase-labeled cells (Fig. 3*a*), even after a 90-min incubation in cycloheximide-containing medium. Although one polypeptide does disappear from whole cells

(double arrowhead in Fig. 3*d*; compare to Fig. 3*a*) most proteins appear to be stable under these control conditions. Fractionation of control cells into cell bodies (Fig. 3*e*) and flagella (Fig. 3*f*) indicates that both α -3 tubulin and at least one other flagellar protein (arrows, Fig. 3, *e* and *f*) partition principally with flagella. This demonstrates that the differential flagellar labeling attained by pulse-chase labeling (Fig. 3, *b* and *c*) is minimally changed after 90 min under these control conditions (Fig. 3, *e* and *f*); flagellar length does not change appreciably under these control conditions (see Fig. 2).

Cells that have resorbed their flagella (Fig. 3*g*) have a 2-D gel autoradiographic pattern that is similar to both pulse-chase-labeled (Fig. 3*a*) and control cells (Fig. 3*d*). While one protein does disappear from cells that have resorbed their flagella (single arrowhead, Fig. 3*g*; compare with Fig. 3, *a* and *d*), the protein that disappeared from control cells remains unchanged by flagellar resorption (compare double arrowheads in Fig. 3, *a*, *d*, and *g*). Cells that have resorbed their flagella (Fig. 3*g*) do differ dramatically from control cells (Fig. 3*d*) in their α -tubulin. While the α -3 tubulin, which is prominently labeled in pulse-chase-labeled cells (Fig. 3*a*), remains unaltered by control conditions (Fig. 3*d*), it is greatly diminished in cells that have resorbed their flagella (Fig. 3*g*). Although these resorption conditions result in cells that do not have flagella when viewed by phase-contrast microscopy, small flagellar stubs actually remain on these cells. Fractionation of these cells that have resorbed their flagella into cell bodies (Fig. 3*h*) and short flagella (Fig. 3*i*) indicates that most of the tubulin, as well as the other flagellar polypeptide which we have analyzed (arrow, Fig. 3, *h* and *i*), partition with the cell body. This indicates that flagellar polypeptides, derived from disassembled flagella, actually appear in the cell body during flagellar resorption. The loss of α -3 tubulin from cells that have resorbed their flagella seemed to be associated with a rise in the amount of α -1 tubulin (compare Fig. 3, *d* and *g*). This rise in α -1 tubulin associated with flagellar resorption, which was especially apparent in shorter autoradiographic exposures (data not shown), was demonstrated by quantitating the tubulin present in 2-D gels (see Materials and Methods). These data (Table I) indicate that flagellar resorption is associated with a substantial increase in α -1 tubulin concomitant with a decrease in α -3 tubulin.

The changes in α -1 and α -3 tubulin abundance that are associated with flagellar resorption are in striking contrast to the behavior of β -tubulin in these experiments. Total α -tubulin is present in equimolar quantities with β -tubulin in many cell types (e.g., 7, 24), including *Chlamydomonas* (data not shown), and any nonspecific change in α -tubulin (e.g., proteolysis) would probably also occur in β -tubulin. We find that abundance of β -tubulin does not change under our experimental conditions (Table I); the elevated levels of ^{35}S that are incorporated into β -tubulin relative to α -tubulin are probably due to more abundant methionine plus cysteine (16, 23, 28, 38, 39, 43). In summary, these results indicate that α -3 tubulin, which is posttranslationally modified from α -1 tubulin during flagellar assembly (18–22, 26), is changed back to α -1 tubulin during flagellar resorption.

DISCUSSION

Recently, we determined that *Chlamydomonas* α -tubulin is posttranslationally modified by acetylation on the epsilon

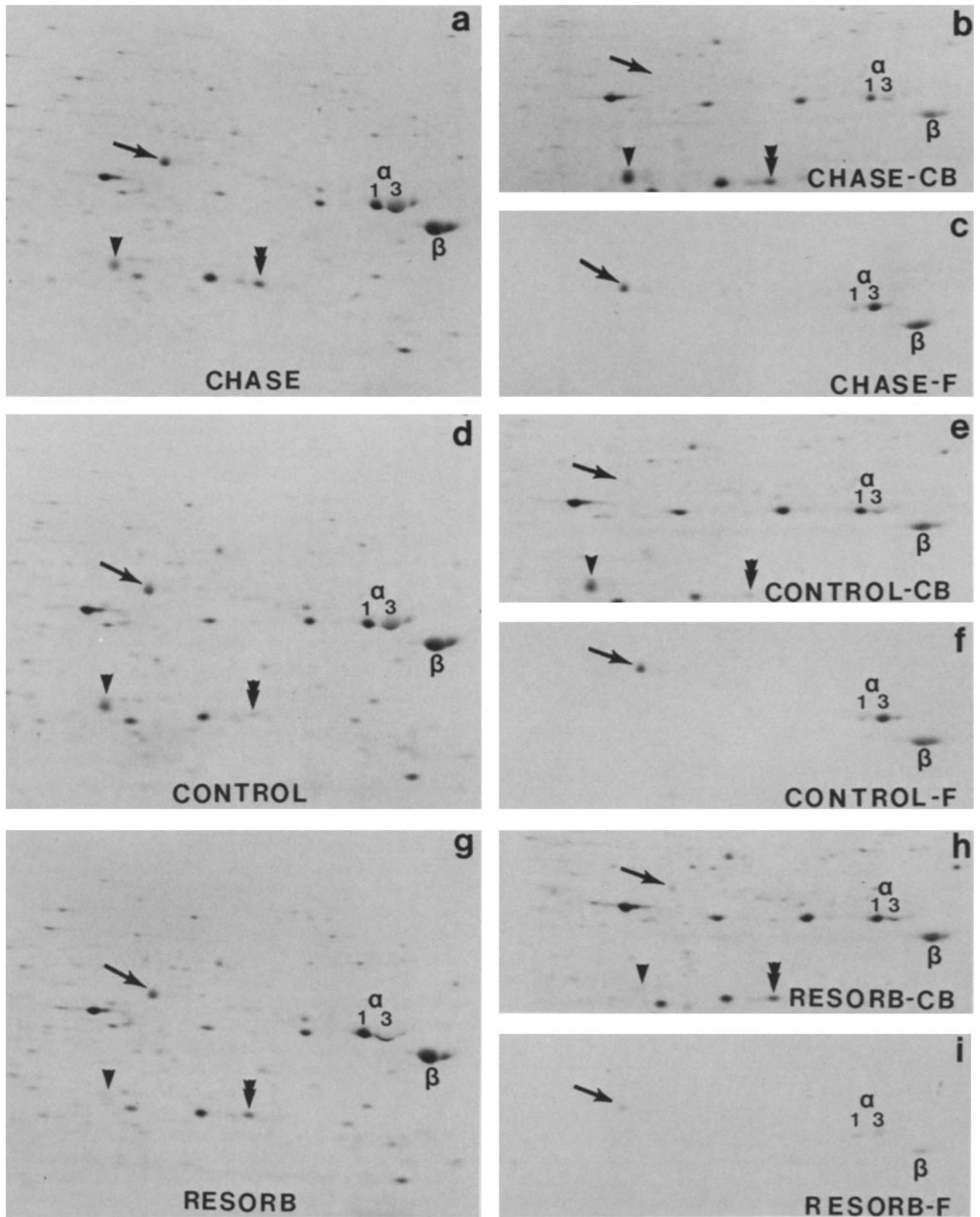


TABLE I
Data

	Counts per minute		
	α -1	α -3	β
Pulse-chase labeling	6,414	10,181	20,481
	<u>6,414</u>	<u>10,000</u>	<u>20,999</u>
	Average- 6,414	10,090	20,740
	39%	61%	
Average Total α -Tubulin—16,504			
Control conditions for flagellar resorption	α -1	α -3	β
	7,545	10,084	20,337
	<u>7,425</u>	<u>9,422</u>	<u>19,158</u>
	Average- 7,485	9,753	19,748
43%	57%		
Average Total α -Tubulin—17,238			
Flagellar resorption	α -1	α -3	β
	10,409	5,199	19,230
	<u>10,722</u>	<u>5,523</u>	<u>20,012</u>
	Average- 10,566	5,361	19,621
66%	34%		
Average Total α -Tubulin—15,927			
Resorb	α -1	α -3	
	10,566	5,361	
Resorb, control	<u>7,485</u>	<u>9,753</u>	
	+3,081	-4,392	

Representative quantitation of α - and β -tubulin after ^{35}S pulse-chase labeling, flagellar resorption, or control conditions for flagellar resorption. Two gels of each sample were run, and each gel was electrophoresed with 1×10^6 cpm of cellular protein derived from $\sim 5.3 \times 10^5$ whole cells (as in Fig. 3, a, d, and g) and quantitated as described in Materials and Methods. Percentages are of total α -tubulin for that sample.

amino group of a lysine(s) during flagellar assembly (22a). The present study indicates that this acetyl group is removed from α -tubulin during flagellar resorption, and, together with previous results (18–22, 26), these data allow an overall interpretation of the relationship of α -tubulin acetylation to the assembly/disassembly of flagellar axonemes. Flagellar amputation causes the movement of α -1 tubulin and its acetylated form from their cell body storage site into the flagella. Both α -1 tubulin and the acetylase migrate through the flagellar matrix fraction (the proteins between the membrane and axoneme [21, 44]) toward the flagellar tip, which is the site of axonemal assembly (2, 4, 12, 32, 45). At a point either just before or after its addition to the growing axoneme, the acetylase cata-

lyzes the posttranslational modification of α -1 to α -3 tubulin. Although the axoneme does contain a small amount of α -1 tubulin ($\sim 20\%$), analysis of mutants (1) and selective axonemal microtubule solubilization shows that this α -1 tubulin is found in all three types of axonemal microtubules (L'Hernault and Rosenbaum, unpublished observations). The process of acetylation is reversed when the flagellar axoneme begins to disassemble during flagellar resorption. This, apparently, occurs from the flagellar tip since the continuity of flagellar microtubules with the basal body is not lost during flagellar resorption (19). A deacetylating enzyme presumably removes the acetyl group from α -3 tubulin-containing dimer during axonemal disassembly. At present, we are not certain whether deacetylation occurs before or at the time the α -tubulin enters the cell body. Nonetheless, tubulin derived from axonemes does accumulate within the cell body during flagellar resorption.

Posttranslational modification of tubulin has been demonstrated in various organisms, and previously identified types of modification include phosphorylation, tyrosinolation (reviewed in references 27 and 47), and acetylation (22, 22a). There is no evidence for either a functional role or subcellular localization of phosphorylated tubulin. Tyrosinolation of α -tubulin, which occurs in a large number of organisms, has been suggested to influence the assembly/disassembly of tubulin (reviewed in reference 42). While most efforts to localize tyrosinolated tubulin to specific organelles or cellular regions have yielded equivocal results, there is one case (*Trypanosoma rhodesiense*) where tyrosinolated α -tubulin is known to occur only within flagella (11). To date, acetylation of α -tubulin has been demonstrated only in *Chlamydomonas*. However, *Crithidia* (33, 34) and *Polytomella* (26) both exhibit flagellar assembly-dependent α -tubulin posttranslational modifications that are remarkably similar to *Chlamydomonas*. Perhaps further analysis of these organisms will demonstrate that they have acetylated α -tubulin.

While the occurrence of in vivo protein acetylation is widespread, the functional implications of these posttranslational modifications are not yet known (47). The reversibility of *Chlamydomonas* α -tubulin acetylation, which occurs during flagellar resorption, suggests that this posttranslational modification has an important function in the assembly of axonemal tubulin. It is also possible that acetylation of α -tubulin might permit the binding of accessory flagellar proteins, such as dynein, nexin, or radial spokes, to flagellar microtubules. It might be involved in the specification of

FIGURE 3 2-D electrophoresis of ^{35}S -labeled *Chlamydomonas* proteins. Cells were pulse-chase-labeled during flagellar regeneration and either prepared for electrophoresis immediately (a) or subjected to electrophoresis after remaining under control (d) or flagellar resorption (g) conditions (see Fig. 1 and Results). Aliquots of these same cells (as in a, d, and g) were fractionated into cell bodies (CB) and flagella (F) and the 2-D gels of these fractions of each cell population appear in the right half of the figure. Each 2-D gel of total cellular protein (a, d, and g) was loaded with 1×10^6 cpm ^{35}S -labeled protein derived from approximately 5.3×10^5 cells. Equal numbers of ^{35}S pulse-chase-labeled, control, and resorbed cells were fractionated into cell bodies and flagella. All flagella or cell body protein pellets were resuspended in equal volumes of lysis buffer. An equal aliquot of each cell body or flagellar sample (which, presumably, contained equal numbers of cell bodies or flagella) was subjected to 2-D electrophoresis. These equal aliquots do not contain equal amounts of radioactive protein because flagellar resorption is associated with the appearance of radioactive flagellar proteins in the cell body (see Fig. 1 and Results). 2-D gel loadings of cell fractions, in terms of counts per minute (cpm) were 9.6×10^5 (b and e), 4×10^4 (c and f), 9.9×10^5 (h), and 1×10^4 (i). All samples were co-electrophoresed with 25 μg of nonradioactive *Chlamydomonas* axonemes and resulting 2-D gels were dried and autoradiographed for 24 h; only a portion of each autoradiograph is shown in this figure. (Arrow) A flagellar protein that does not appear to have a significant cytoplasmic pool. (Arrowhead) A nonflagellar protein that specifically disappears from cells that have resorbed their flagella. (Double arrowhead) A nonflagellar protein whose disappearance is inhibited by flagellar resorption conditions.

tubulin polymerization into outer doublet microtubules. Alternatively, acetylation might release tubulin from conditions that restrict its assembly, such as the binding of a polymerization-limiting protein analogous to the actin-binding protein profilin (8, 40, 41; see review in reference 15).

Another possibility is that α -tubulin acetylation might allow the use of a common tubulin for more than one microtubular structure. *Chlamydomonas* contains at least four other microtubular structures (cytoplasmic microtubules, mitotic apparatus, basal bodies, and cleavage furrow microtubules) in addition to flagella (9, 10, 13, 14, 29, 32) but contains only two α - and two β -tubulin genes (6, 36, 37). Additional functional diversity could be obtained by posttranslational modification. For example, in the related alga *Polytomella*, α -tubulin in the cytoplasmic microtubules is principally α -1, while axonemes contain mainly α -3 tubulin (25) and evidence indicates that α -1 tubulin is a precursor to α -3 tubulin (26). Flagella are resorbed by *Chlamydomonas* before the elaboration of several different types of nonflagellar microtubules associated with mitosis and division (9, 10, 14, 29). Since the biophysical characteristics of flagellar and nonflagellar microtubules are quite different (3), a reversible posttranslational modification might confer the necessary biophysical differences on a common gene product and permit its use in several different *Chlamydomonas* microtubular structures.

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