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: 35SO4 (as H235SO4, 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 35S-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 μCi/ml 35S 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) for 30 min. Cells were resuspended in a modified medium 1 (35). This modified medium 1 lacked KH2PO4, contained 5 mM Na2SO4 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 μg/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 μg/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) gels that had been loaded with an equal amount of protein. Other materials were as described previously (21, 44, 46).

Abbreviations 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 fractions 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 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 R in a tightly sealed vial at 55°C for 24 h.

Samples were cooled to room temperature, 10 ml of Liquiflour R 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 medium 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 (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 flagellless (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 autoradiographed (17) as described previously.
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. 3a). The principal $\alpha$-tubulin observed under these conditions was $\alpha$-3, which has previously been shown to be the principal flagellar $\alpha$-tubulin (18-22, 26). When pulse-chase labeled cells (as in Fig. 3a) were fractionated into cell bodies (Fig. 3b, e, and h) and flagella (Fig. 3c), 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. 3a, b, and c) that is incorporated into $\beta$-tubulin relative to $\alpha$-tubulin are in striking contrast to many cell types (e.g., 7, 24), including Chlamydomonas (data not shown), and any nonspecific change in $\alpha$-tubulin (e.g., proteolysis) would probably also occur in $\beta$-tubulin. We find that abundance of $\beta$-tubulin does not change under our experimental conditions (Table I); the elevated levels of $^{35}$S that are incorporated into $\beta$-tubulin relative to $\alpha$-tubulin are probably due to more abundant methionine plus cysteine (16, 23, 28, 38, 39, 43). In summary, these results indicate that $\alpha$-3 tubulin, which is posttranslationally modified from $\alpha$-1 tubulin during flagellar assembly (18-22, 26), is changed back to $\alpha$-1 tubulin during flagellar resorption.

DISCUSSION

Recently, we determined that Chlamydomonas $\alpha$-tubulin is posttranslationally modified by acetylation on the epsilon

**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.
Control conditions for flagellar or flagellar resorption (g) conditions (see Fig. 1 and Results). Aliquots of these same cells (as in a, d, and g) were fractionated into flagella. All flagella or cell body protein pellets were resuspended in equal volumes of lysis buffer. An equal aliquot of each cell 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.

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 x 10⁶ (b and e), 4 x 10⁷ (c and f), 9.9 x 10⁶ (h), and 1 x 10⁷ (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.

### TABLE 1

<table>
<thead>
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<th>Data</th>
<th>α-1</th>
<th>α-3</th>
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<td>10,722</td>
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Representative quantitation of α- and β-tubulin after 35S 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 x 10⁶ cpm of cellular protein derived from ~5.3 x 10⁶ 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.
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 microtubular structures (cytoplasmic microtubules, motile 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 could confer the necessary biophysical differences on a common gene product and permit its use in several different *Chlamydomonas* microtubular structures.

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REFERENCES


