α-Tubulin Acetylase Activity in Isolated *Chlamydomonas* Flagella

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ABSTRACT We have previously shown that the α-tubulin of *Chlamydomonas* flagella is synthesized as a precursor which is modified by acetylation in the flagellum during flagellar assembly. In this report, we show the presence of an α-tubulin acetylase activity in isolated *Chlamydomonas* flagella that is highly specific for α-tubulin of both mammalian brain and *Chlamydomonas*.

Detachment of the flagella of the bi-flagellate alga *Chlamydomonas* stimulates the synthesis and accumulation of mRNAs for tubulin and other flagellar proteins (1–4). Translation of these mRNAs in a reticulocyte lysate system showed the flagellar α-tubulin to be made as a precursor (α_1), slightly more basic than the mature α-tubulin (α_3) found in the assembled microtubules of flagella (5–7). The α-tubulin modification was identified as an acetylation by inhibiting protein synthesis while allowing the flagella to regenerate in the presence of radioactive acetate. Isolation of these flagella and two-dimensional gel electrophoretic analysis indicated that, of more than 150 flagellar proteins, only α-tubulin became labeled (8), and that this labeling was due to an acetylation of the ϵ-amino group of lysine (9). If flagellar assembly was completely inhibited by colchicine, flagellar α-tubulin precursor (α_1) accumulated in the cell body (10, 11); upon release from colchicine inhibition of assembly, the α_1 precursor was then found in the detergent-soluble "matrix" of the flagellum (10), presumably on its way to the tip assembly site (12–14). Thus, flagellar α-tubulin is made as a precursor which can be found in the cell body; it moves up the shaft of the flagellum as a precursor and it is changed by acetylation to the mature α_3-tubulin, either just prior to or at the time of microtubule assembly at the flagellar tip. Because of these results, it was reasonable to analyze isolated flagella for α-tubulin acetylase activity. The following report shows that this enzymatic activity is present in the flagella and that it specifically acetylates α-tubulin of *Chlamydomonas* and mammalian brain.

MATERIALS AND METHODS

Materials: [^{3}H]-Acetyl-Coenzyme A (CoA)[1] was obtained from ICN Radiochemicals (Irvine, CA) at a specific activity of 3.4 Ci/mmol, or from Amersham Corp. (Arlington Heights, IL) at a specific activity of 1.5 Ci/mmol. Dithiothreitol and guanosine 5′-triphosphate (GTP) were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN) and acetyl-CoA from Calbiochem-Behring Corp. (San Diego, CA). Nonidet P-40 was obtained from Sigma Chemical Co. (St. Louis, MO), and Liquifluor from New England Nuclear (Boston, MA).

Cell Culture and Fractionation: Wild-type *Chlamydomonas reinhardtii*, strain 21gr vegetative cells, were grown in Medium I, or in acetate-containing Medium II plus five times the normal concentration of phosphorus (15). *Chlamydomonas* flagella, axonemes, and membrane/matrix fractions were prepared as described previously (16, 17).

Preparation of Brain Microtubule Protein: Microtubule protein was prepared from calf brain by the method of Sloboda et al. (18) and stored as a pellet at −80°C. Before use in an acetylation assay, the microtubule protein was thawed in a 37°C water bath with 0.5 mM fresh GTP added (final GTP concentration 2.5 mM) and carried through a final cycle of temperature-dependent polymerization/depolymerization in PM buffer (100 mM PIPES, pH 6.9, with KOH, 1 mM $MgSO_4$, 2 mM GTP, and 2 mM EGTA). For some experiments, PM buffer contained 4 M glycerol. Purified porcine tubulin was prepared by phosphocellulose chromatography (18).

Assay for In Vitro Acetylation of α-Tubulin: Acetylation of α-tubulin was assayed by measuring the incorporation of tritium from [^{3}H]-acetyl-CoA into trichloroacetic acid–precipitable protein. In a typical reaction, brain microtubule protein at 2–4 mg/ml in PM buffer was mixed with an equal volume of flagella at 1–4 mg/ml in PM buffer. For some experiments, flagella were added in the buffer used for flagellar isolation which contained 10 mM HEPES, pH 7.5, 5 mM $MgSO_4$, 1 mM dithiothreitol, and 4% sucrose. The flagella were permeabilized with 0.1% Nonidet P-40 before addition to the assay. We have found that this permeabilization is unnecessary in the presence of 4 M glycerol, but is required in buffers such as the aforementioned HEPES/$MgSO_4$/dithiothreitol/sucrose buffer, which leave the flagellar membrane intact (16). Reactions were started by the addition of [^{3}H]-acetyl-CoA, usually 10 μCi/ml, and were carried out at 37°C. To determine incorporation of radioactivity from [^{3}H]-acetyl-CoA, 50-μl samples were removed from the reaction and placed on Whatman 3 MM filter paper disks (Whatman Inc., Clifton, NJ), then immediately dropped into ice-cold 10% trichloroacetic acid. The disks were processed for liquid scintillation counting (19) and counted in Liquifluor.

Other Methods: Protein determinations were performed by the method of Bradford (20), using chicken ovalbumin as a standard. Standard

[1] *Abbreviations used in this paper:* CoA, Coenzyme A; GTP, guanosine 5′-triphosphate; PM buffer, 100 mM PIPES, pH 6.9, with KOH, 1 mM $MgSO_4$, 2 mM GTP, and 2 mM EGTA.

methods for one-dimensional (21) and two-dimensional (22) PAGE and fluorography of gels (23) were used, with modifications as previously described (8, 15).

RESULTS

Acetylation of Calf Brain α-Tubulin by Flagellar Acetylase

The Coomassie Blue–stained SDS polyacrylamide gel (Fig. 1, *a–c*) shows that the in vitro–assembled calf brain microtubule protein used as a substrate for the flagellar acetylase contains the prominent α- and β-tubulins and the high molecular weight microtubule-associated proteins in addition to many other proteins present in smaller amounts (Fig. 1 *b*). *Chlamydomonas* flagella, the acetylase source, also contain many proteins in addition to the α- and β-tubulins of the intact axonemes (Fig. 1 *c*). The mixture of brain microtubule protein and flagella is shown in Fig. 1 *a*. When brain microtubule protein is incubated with flagella in the presence of [^{3}H]-acetyl-CoA, α-tubulin is the only protein substantially labeled (Fig. 1 *d*, fluorograph), although many other proteins are present. Equally exposed fluorographs of brain microtubule protein alone (Fig. 1 *e*) or flagella alone (Fig. 1 *f*) incubated with [^{3}H]-acetyl-CoA show no labeling of α-tubulin.

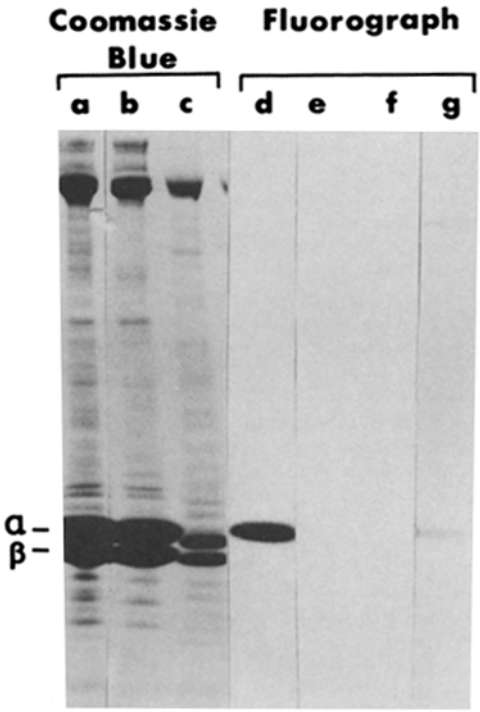

FIGURE 1 Acetylation of calf brain microtubule protein in vitro. Coomassie Blue–stained gel (*a–c*) and fluorograph exposed for 5 d (*d–f*). Twice-cycled calf brain microtubule protein plus isolated whole *Chlamydomonas* flagella (*a* and *d*); twice-cycled calf brain microtubule protein alone (*b* and *e*); isolated whole *Chlamydomonas* flagella alone (*c* and *f*). Incubations were with 10 μCi/ml [^{3}H]-acetyl-CoA (2.9 μM) for 40 min at 37°C. The concentration of brain microtubule protein was 4 mg/ml, and of flagellar protein, 2 mg/ml. In these fluorographs, acetylation of α-tubulin is seen only in the mixture of brain and flagellar protein (*d*), and not in either of the two preparations incubated separately (*e* and *f*). A fluorograph of brain microtubule protein exposed for 11 d shows some endogenous acetylation of α-tubulin (*g*) that is undetectable in the shorter exposure (*e*).

However, with longer exposure times (Fig. 1 *g*), a small amount of endogenous α-tubulin acetylase can be seen in the brain microtubule preparation. Brain tubulin purified by phosphocellulose chromatography has no detectable endogenous acetylase, yet the α-tubulin of this preparation is readily and specifically labeled in the presence of flagellar protein (data not shown). One can assay for the α-tubulin acetylase simply by trichloroacetic acid precipitation of total protein of the incubation mixture, since the α-tubulin of brain microtubule protein is the principal substrate in an incubation mixture containing both brain and flagellar proteins. Flagella and brain microtubule protein incubated separately incorporate <15% of the radioactivity precipitated from the total incubation mixture, and this can be subtracted to obtain the flagellar acetylase activity. These results indicate, therefore, that isolated *Chlamydomonas* flagella contain an active tubulin acetylase which, in the presence of many different flagellar and brain microtubule proteins, will specifically acetylate only α-tubulin. The maximal acetylation we have observed is 1.1 mol acetate/mol α-tubulin.

Acetylation of Flagellar α-Tubulin by the Flagellar Acetylase

Although the α-tubulin acetylase of *Chlamydomonas* flagella can use brain α-tubulin as a substrate, its endogenous substrate should be *Chlamydomonas* α_1-tubulin, which we have shown by in vivo labeling experiments to be the unacetylated soluble precursor of the acetylated α_3-tubulin of flagellar axonemal microtubules (6–8). The flagella used as an enzyme source for the experiment illustrated in Fig. 1 actually contain a small percentage of this α_1 precursor in their detergent-soluble "matrix" fraction, but the great majority of the flagellar tubulin is in the already acetylated α_3 form of the assembled axonemes (8). Therefore, the fluorograph exposure time for the gel shown in Fig. 1 *e* is too short to observe any acetylation of this small amount of endogenous α_1-tubulin. However, if the exposure times for the fluorographs are greatly increased, one can observe the acetylation of endogenous α-tubulin in the isolated flagella as shown on a one-dimensional gel (Fig. 2 *a*) and fluorograph (2 *b*). That this α-tubulin labeling is the acetylation of α_1-tubulin to form the acetylated α_3-tubulin is indicated in Fig. 2, *c* and *d*, which illustrate two-dimensional gel analysis of the same preparation. The two tubulins are separated into the typical α_1-, α_3-, and β-tubulins in the Coomassie Blue–stained gel, with the α_3-tubulin of the assembled axoneme greatly predominating, as we have shown earlier (8). The fluorograph of this gel shows that only the α_3-tubulin is labeled. Therefore, the *Chlamydomonas* α-tubulin acetylase is capable of acetylating the endogenous α_1-tubulin present in isolated flagella.

DISCUSSION

The work reported here shows that isolated *Chlamydomonas* flagella contain an acetylase activity that will specifically acetylate the α-tubulin of brain and *Chlamydomonas* flagellar microtubule precursor protein. The enzyme is probably functional only at the distal tip of the flagellum because (*a*) all flagellar microtubule assembly takes place at the distal tip of the flagellum (12–14), (*b*) almost all of the α-tubulin in the assembled flagellar axoneme is in the acetylated, α_3 form (8), and (*c*) the unassembled and unacetylated α_1-tubulin can be

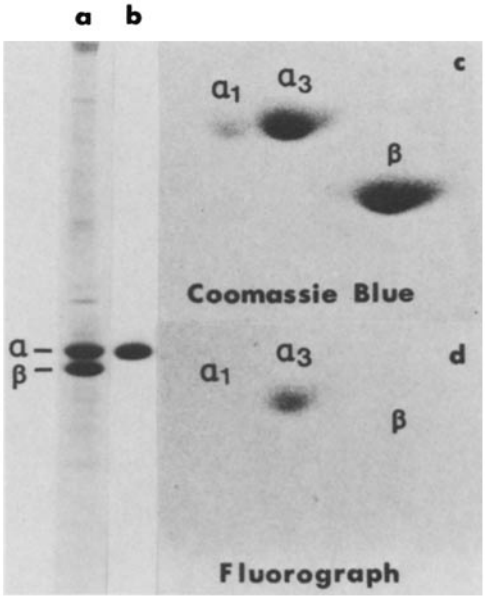

FIGURE 2 Acetylation of endogenous *Chlamydomonas* α-tubulin. (*a*) Coomassie Blue–stained one-dimensional gel and (*b*) fluorograph of the gel in *a* exposed for 16 d. This long exposure shows endogenous acetylation of flagellar α-tubulin of the whole *Chlamydomonas* flagella. (*c*) The tubulin region of a Coomassie Blue–stained two-dimensional gel shows α_1-, α_3-, and β-tubulin. (*d*) A fluorograph of *c* shows that some of the α_3-tubulin was formed by acetylation in vitro.

found in the detergent-soluble "matrix" compartment of the flagellum (8), presumably on its way to the tip assembly site. The mechanism that confines acetylated α-tubulin to the flagellum appears to be more complex than flagellar compartmentalization of the acetylase or its substrate. The initial in vivo labeling experiments that were done to show that α_1-tubulin became acetylated during flagellar regeneration also indicated that acetylase was present in the cell body as well as the flagellum. Thus, if *Chlamydomonas* flagella were detached in cycloheximide so that protein synthesis was inhibited, the flagella assembled to half-length (13), and their microtubules contained acetylated α-tubulin (5–8). Therefore, the acetylase and the α-tubulin substrate had to be present in the cell body prior to flagellar regeneration. The reasons why the acetylase is functional only at the flagellar distal tip, where microtubule assembly takes place (12–14), even though both α_1-tubulin and the acetylase are present in the cell body and the "matrix" fraction of the flagellum, are not known. Preliminary results suggest the presence of an inhibitor and a deacetylase in *Chlamydomonas* cell bodies (unpublished data). The site of posttranslational modification in the cell might be regulated either by localized inhibition of the modifying enzyme, or by the presence of a localized deacetylating activity. The answer to this problem will probably emerge from studies on the purified acetylase, the deacetylation enzyme, and the enzyme inhibitor, and their affinity for each other or for tubulin in its different assembly states.

The ability to acetylate α-tubulin in vitro is an aid to the investigation of the functional significance of this modification in *Chlamydomonas*, where acetylated α-tubulin predominates only in the axoneme (5–8). An assembled axoneme differs in many ways from its precursor proteins and from cytoplasmic microtubules. Thus, there are many possible roles in the regulation of flagellar biogenesis for this reversible (24) acetylation. These include enhancement of microtubular stability and interaction with proteins such as dynein and the radial spokes. Since such properties distinguish the axonemal microtubules of all flagellated cells, α-tubulin modification is unlikely to occur only in *Chlamydomonas*. Indeed, flagellar-specific α-tubulins that are more acidic than their cytoplasmic counterparts and appear to arise by posttranslational modification have been found not only in *Polytomella*, a close relative of *Chlamydomonas* (25), but also in the slime mold *Physarum* (26) and the trypanosome *Crithidia* (27, 28). Although acetylation of α-tubulin has not yet been demonstrated in any of these, the appearance of flagellar-specific α-tubulin in organisms that are evolutionarily distant from *Chlamydomonas* suggests a fundamental role for the posttranslational generation of tubulin diversity.

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NOTE ADDED IN PROOF: The reader is directed to the following report by G. Piperno and M. Fuller in which monoclonal antibodies specific for the acetylated form of α-tubulin have been used to show the presence of acetylated α-tubulin in the cilia and flagella of several different cell types

REFERENCES

1. Silflow, C. D., and J. L. Rosenbaum. 1981. Multiple α- and β-tubulin genes in *Chlamydomonas* and regulation of tubulin mRNA levels after deflagellation. *Cell.* 24:81–88.
2. Minami, S. A., P. S. Collis, E. E. Young, and D. P. Weeks. 1981. Tubulin induction in *C. reinhardii*: requirement for tubulin mRNA synthesis. *Cell.* 24:89–95.
3. Schloss, J. A., C. D. Silflow, and J. L. Rosenbaum. 1984. mRNA abundance changes during flagellar regeneration in *Chlamydomonas reinhardtii*. *Mol. Cell Biol.* 4:424–434.
4. Baker, E. J., J. A. Schloss, and J. L. Rosenbaum. 1984. Rapid changes in tubulin RNA synthesis and stability induced by deflagellation in *Chlamydomonas*. *J. Cell Biol.* 99:2074–2081.
5. Lefebvre, P. A., C. D. Silflow, E. D. Wieben, and J. L. Rosenbaum. 1980. Increased levels of mRNAs for tubulin and other flagellar proteins after amputation or shortening of *Chlamydomonas* flagella. *Cell.* 20:469–477.
6. Silflow, C. D., P. A. Lefebvre, T. W. McKeithan, J. A. Schloss, L. R. Keller, and J. L. Rosenbaum. 1981. Expression of flagellar protein genes during flagellar regeneration in *Chlamydomonas*. *Cold Spring Harbor Symp. Quant. Biol.* 46:157–169.
7. McKeithan, T. W., P. A. Lefebvre, C. D. Silflow, and J. L. Rosenbaum. 1983. Multiple forms of tubulin in *Polytomella* and *Chlamydomonas*: evidence for a precursor of flagellar α-tubulin. *J. Cell Biol.* 96:1056–1063.
8. L'Hernault, S. W., and J. L. Rosenbaum. 1983. *Chlamydomonas* α-tubulin is posttranslationally modified in the flagella during flagellar assembly. *J. Cell Biol.* 97:258–263.
9. L'Hernault, S. W., and J. L. Rosenbaum. 1985. *Chlamydomonas* α-tubulin is posttranslationally modified by acetylation on the ε-amino group of a lysine. *Biochemistry.* 24:473–478.
10. L'Hernault, S. W. 1984. Identification and control of a reversible alpha tubulin post-translational modification in *Chlamydomonas*. Ph.D. thesis. Yale University, New Haven, CT. 139 pp.
11. Brunke, K. J., Collis, P. S., and D. P. Weeks. 1982. Post-translational modification of tubulin dependent on organelle assembly. *Nature (Lond.).* 297:516–518.
12. Rosenbaum, J. L., and F. M. Child. 1967. Flagellar regeneration in protozoan flagellates. *J. Cell Biol.* 34:345–364.
13. Rosenbaum, J. L., J. E. Moulder, and D. L. Ringo. 1969. Flagellar elongation and shortening in *Chlamydomonas*: the use of cycloheximide and colchicine to study the synthesis and assembly of flagellar proteins. *J. Cell Biol.* 41:600–619.
14. Witman, G. B. 1975. The site of *in vivo* assembly of flagellar microtubules. *Ann. N.Y. Acad. Sci.* 253:178–191.
15. Lefebvre, P. A., S. A. Nordstrom, J. E. Moulder, and J. L. Rosenbaum. 1978. Flagellar elongation and shortening in *Chlamydomonas*. IV. Effects of flagellar detachment, regeneration, and resorption on the induction of flagellar protein synthesis. *J. Cell Biol.* 78:8–27.
16. Witman, G. B., J. Plummer, and G. Sander. 1978. *Chlamydomonas* flagellar mutants lacking radial spokes and central tubules: structure, composition, and function of specific axonemal compartments. *J. Cell Biol.* 76:729–747.
17. Witman, G. B., K. Carlson, J. Berliner, and J. L. Rosenbaum. 1972. *Chlamydomonas* flagella. I. Isolation and electrophoretic analysis of microtubules, matrix, membranes,

and mastigonemes. *J. Cell Biol.* 54:507–539.
18. Sloboda, R. D., W. L. Dentler, and J. L. Rosenbaum. 1976. Microtubule-associated proteins and the stimulation of tubulin assembly *in vitro*. *Biochemistry.* 15:4497–4505.
19. Mans, R. J., and G. D. Novelli. 1961. Measurement of the incorporation of radioactive amino acids into protein by a filter-paper disk method. *Arch. Biochem. Biophys.* 94:48–53.
20. Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* 72:248–254.
21. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature (Lond.)* 227:680–685.
22. O'Farrell, P. H. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250:4007–4021.
23. Laskey, R. A., and A. D. Mills. 1975. Quantitative film detection of ^3H and ^{14}C in polyacrylamide gels by fluorography. *Eur. J. Biochem.* 56:335–341.
24. L'Hernault, S. W., and J. L. Rosenbaum. 1985. Reversal of the posttranslational modification on *Chlamydomonas* flagellar α-tubulin occurs during flagellar resorption. *J. Cell Biol.* 100:457–462.
25. McKeithan, T. W., and J. L. Rosenbaum. 1981. Multiple forms of tubulin in the cytoskeletal and flagellar microtubules of *Polytomella*. *J. Cell Biol.* 91:352–360.
26. Burland, T. G., K. Gull, T. Schedl, R. S. Boston, and W. F. Dove. 1983. Cell type-dependent expression of tubulins in *Physarum*. *J. Cell Biol.* 97:1852–1859.
27. Russell, D. G., D. Miller, and K. Gull. 1984. Tubulin heterogeneity in the trypanosome *Crithidia fasciculata*. *Mol. Cell. Biol.* 4:779–790.
28. Russell, D. G., and K. Gull. 1984. Flagellar regeneration of the trypanosome *Crithidia fasciculata* involves post-translational modification of cytoplasmic alpha tubulin. *Mol. Cell. Biol.* 4:1182–1185.