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 (αi), slightly more basic than the mature α-tubulin (α3) 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 (αi) accumulated in the cell body (10, 11); upon release from colchicine inhibition of assembly, the αi 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 α3-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 acetylation 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: [3H]-Acetyl-Coenzyme A (CoA) 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).

Preparation of Brain Microtubule Protein: Microtubule protein was prepared from calf brain by the method of Blobel 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/denpolymerization in PM buffer (100 mM PIPES, pH 6.9, with KCl, 1 mM MgSO4, 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 [3H]-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 MgSO4, 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/MgSO4/dithiothreitol/sucrose buffer, which leave the flagellar membrane intact (16). Reactions were started by the addition of [3H]-acetyl-CoA, usually 10 μCi/ml, and were carried out at 37°C. To determine incorporation of radioactivity from [3H]-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
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. 1c). The mixture of brain microtubule protein and flagella is shown in Fig. 1a. When brain microtubule protein is incubated with flagella in the presence of [3H]-acetyl-CoA, α-tubulin is the only protein substantially labeled (Fig. 1d, fluorograph), although many other proteins are present. Equally exposed fluorographs of brain microtubule protein alone (Fig. 1e) or flagella alone (Fig. 1f) incubated with [3H]-acetyl-CoA show no labeling of α-tubulin.

However, with longer exposure times (Fig. 1g), a small amount of endogenous α-tubulin acetylation 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.

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 [3H]-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).

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, α3 form (8), and (c) the unassembled and unacetylated α2-tubulin can be
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 \(\alpha\)-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 \(\alpha\)-tubulin became acetylated during flagellar regeneration also indicated that acetylation was present in the cell body as well as the flagellum. Thus, if \textit{Chlamydomonas} flagella were detached in cycloheximide so that protein synthesis was inhibited, the flagella assembled to half-length (13), and their microtubules contained acetylated \(\alpha\)-tubulin (5–8). Therefore, the acetylation and the \(\alpha\)-tubulin substrate had to be present in the cell body prior to flagellar regeneration. The reasons why the acetylation is functional only at the flagellar distal tip, where microtubule assembly takes place (12–14), even though both \(\alpha\)-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 \textit{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 deacetylating enzyme, and the enzyme inhibitor, and their affinity for each other or for tubulin in its different assembly states.

The ability to acetylate \(\alpha\)-tubulin in vitro is an aid to the investigation of the functional significance of this modification in \textit{Chlamydomonas}, where acetylated \(\alpha\)-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, \(\alpha\)-tubulin modification is unlikely to occur only in \textit{Chlamydomonas}. Indeed, flagellar-specific \(\alpha\)-tubulins that are more acidic than their cytoplasmic counterparts and appear to arise by posttranslational modification have been found not only in \textit{Polychromella}, a close relative of \textit{Chlamydomonas} (25), but also in the slime mold \textit{Physarum} (26) and the trypanosome \textit{Crithidia} (27, 28). Although acetylation of \(\alpha\)-tubulin has not yet been demonstrated in any of these, the appearance of flagellar-specific \(\alpha\)-tubulin in organisms that are evolutionarily distant from \textit{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 \(\alpha\)-tubulin have been used to show the presence of acetylated \(\alpha\)-tubulin in the cilia and flagella of several different cell types.

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