Acetylated α-Tubulin in *Physarum*: Immunological Characterization of the Isotype and Its Usage in Particular Microtubular Organelles

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Abstract. We have used monoclonal antibodies specific for acetylated and unacetylated α-tubulin to characterize the acetylated α-tubulin isotype of *Physarum polycephalum*, its expression in the life cycle, and its localization in particular microtubular organelles. We have used the monoclonal antibody 6-11B-1 (Piperno, G., and M. T. Fuller, 1985, *J. Cell Biol.*, 101:2085–2094) as the probe for acetylated α-tubulin and have provided a biochemical characterization of the monoclonal antibody KMP-1 as a probe for unacetylated tubulin in *Physarum*. Concomitant use of these two probes has allowed us to characterize the acetylated α-tubulin of *Physarum* as the α3 isotype. We have detected this acetylated α3 tubulin isotype in both the flagellate and in the myxameba, but not in the plasmodium. In the flagellate, acetylated tubulin is present in both the flagellar axonemes and in an extensive array of cytoplasmic microtubules. The extensive arrangement of acetylated cytoplasmic microtubules and the flagellar axonemes are elaborated during the myxameba–flagellate transformation. In the myxameba, acetylated tubulin is not present in the cytoplasmic microtubules nor in the mitotic spindle microtubules, but is associated with the two centrioles of this cell. These findings, taken together with the apparent absence of acetylated α-tubulin in the ephemeral microtubules of the plasmodium suggest a natural correspondence between the presence of acetylated α-tubulin and microtubule organelles that are intrinsically stable or cross-linked.

IN many organisms there is now excellent evidence for the existence of multi-gene families encoding both α- and β-tubulin. The organization and number of the tubulin genes varies between organisms and it is clear that in many cases there is a complex pattern of differential expression that leads to the appearance of different tubulin isotypes in particular cell types or tissues (6, 25). There is also good evidence that expression of these tubulin multi-gene families can lead to the presence of multiple α- or β-tubulin polypeptides within individual cells. However, at least in the case of α-tubulin, this is not the only method by which an individual cell can provide itself with a variety of tubulin isotypes. Two well-defined, posttranslational modifications have been described that produce alternative isotypes of α-tubulin. The α-tubulin polypeptide can undergo both acetylation and detyrosination; in both cases the posttranslational modification appears reversible to produce, respectively, the original unacetylated and tyrosinated form of the polypeptide (1, 15–17, 30).

The posttranslational acetylation of α-tubulin was discovered in the unicellular green alga *Chlamydomonas reinhardtii*. In this and other organisms such as trypanosomes (20, 28, 29), the major α-tubulin detected in the flagellum possesses unique electrophoretic coordinates on a two-dimensional gel. This particular α-tubulin isotype (α3) is formed via a posttranslational event from the α-tubulin (α1) encoded by the tubulin mRNA and the modification appears to occur either immediately before or immediately after the inclusion of a tubulin polypeptide into the microtubule. In *Chlamydomonas*, this posttranslational modification has been shown to involve the acetylation of the ε-amino group of a lysine residue in the α-tubulin polypeptide (17). More recently, the enzymatic activity responsible for the acetylation reaction has been identified and characterized in isolated flagella of *Chlamydomonas* (10). The α-tubulin acetylase has been shown to have high specificity for α-tubulin, however the *Chlamydomonas* enzyme will acetylate the α-tubulin of both *Chlamydomonas* and mammalian brain.

An important development in understanding the cellular distribution of acetylated tubulin has come with the recent description of a monoclonal antibody that specifically recognizes this form of α-tubulin (24). The antibody was raised against the tubulin from the axonemes of sea urchin sperm flagella but has been shown to recognize acetylated α-tubulin in cilia and flagella from a variety of organisms. The antigen was not found in the soluble, cytoplasmic pool tubulin, confirming the earlier view of the time of production of the acetylated α-tubulin isotype during microtubule construction in vivo (15, 20). Modification of α-tubulin by acetylation leads to the production of a novel tubulin isotype, the true

function of which is, at present, unknown. In this context we have sought to ascertain whether the acetylated tubulin isotype is present in all cells in the life cycle of an organism and, if present, whether it functions within all of the microtubular organelles constructed by an individual cell. Knowledge of the precise natural distribution of acetylated tubulin is likely to be crucial in understanding how a cell controls its production and usage. We have chosen to use for these experiments the slime mould *Physarum polycephalum* since this organism is known to possess distinct cell types that assemble microtubular organelles of precise arrangement and different function. *Physarum* myxamebas are single cells in which microtubules are present as components of the cytoplasmic cytoskeleton, centrioles, and the open mitotic spindle. The myxameba can undergo a reversible transformation into a nonproliferating flagellate that possesses polymerized tubulin in cytoplasmic microtubules, basal bodies, and the flagellar axonemes. Under appropriate conditions, a myxameba can also transform into a plasmodium. This is a macroscopic, single cell that does not possess cytoplasmic microtubules; tubulin is only polymerized during mitosis in the microtubules of the intranuclear mitotic spindle. The various cell types of the *Physarum* life cycle also express different tubulin isotypes, some being the products of different genes and at least one being formed by a posttranslational modification (5, 9, 27).

We have used two monoclonal antibodies that have enabled us to detect the acetylated and unacetylated isotypes of α-tubulin in *Physarum*. We have been able to show that the α3 tubulin isotype, produced via a posttranslational modification, is acetylated. This acetylated α3 tubulin isotype is a major isotype in the flagellate, but is also present in the myxameba. We have been unable to detect acetylated tubulin in the plasmodial phase of the life cycle. In those cell types that possess acetylated tubulin there is a very precise distribution of the modified tubulin in particular microtubular organelles. In the flagellate, acetylated tubulin is present in both cytoplasmic microtubules as well as those of the flagellum axoneme. In the myxamebal cell, the acetylated tubulin appears to be associated with the two centrioles, whilst the adjacent cytoplasmic microtubules or mitotic spindle microtubules are unacetylated. These findings, taken together with the apparent absence of acetylated tubulin in the ephemeral microtubules of the plasmodium suggest a correspondence between the presence of acetylated α-tubulin and microtubule organelles that are intrinsically stable or cross-linked.

Materials and Methods

Physarum polycephalum Cultures

The strains of *Physarum polycephalum* myxamebas used in this study were CLd, CLd-AXE, and LU 352. LU 352 is a recently isolated strain of amebas which has inherited the ability to grow in liquid media from the mutant strain CLd-AXE (19) but unlike CLd-AXE has the ability to transform into flagellates when transferred to non-nutrient liquid conditions (7). The strain LU 352 was a gift from Dr. J. Dee (University of Leicester, Leicester, England). LU 352 and CLd-AXE were grown at 26°C in a liquid semi-defined medium according to the method of McCullough and Dee (18), modified to include 1% (wt/vol) bacteriological peptone. CLd was grown on *Escherichia coli* lawns on liver infusion agar at 25°C. *Physarum polycephalum* strain CLd × LU 862 was used for micro- and macroplasmodia studies. Plasmodial growth conditions were as described previously (26, 27).

Monoclonal Antibodies

The two main monoclonal antibodies used throughout this study were 6-11B-1 and KMP-1. 6-11B-1 is a mouse monoclonal of the IgG type. It was raised against sea urchin sperm axonemes and reacts with acetylated α-tubulin from a variety of species (24). This antibody was kindly donated by Dr. G. Piperno (The Rockefeller University, New York). KMP-1 is a mouse monoclonal of the IgM type. It was raised against *Physarum* myxamebal tubulin (3). Other general anti-α-tubulin monoclonal antibodies used were YOL1/34 and YL1/2 (13), which were gifts from Dr. J. Kilmartin (Medical Research Council, Cambridge) and DM1A (4), which was a gift from Dr. S. Blose (Cold Spring Harbor Laboratories, Cold Spring Harbor, NY). KMX-1 was used as a general anti-β-tubulin monoclonal (3).

Protein Purification

Microtubule protein was purified from *Physarum* microplasmodial strain CLd × LU862 and myxamebal strain CLd-AXE essentially according to the method described by Roobol et al. (27).

Chemical Acetylation

Myxamebal microtubule protein (1.5 mg; 5 mg/ml) was dialyzed against a half-saturated aqueous solution of sodium acetate at 4°C, then modified by six additions at 5-min intervals, each of 1 μl acetic anhydride (21). Protein and reactants were separated on a G25 column equilibrated with 50 mM Na phosphate, pH 7.0. The protein-containing eluate was made 1 mM in hydroxylamine and kept at 4°C for 30 min, dialyzed against water at 4°C for 24 h, then lyophilized. A second 1.5-mg sample of the microtubule protein (the experimental control) was treated identically except that acetic anhydride was not added.

Electrophoretic Techniques

Samples and IEF/SDS two-dimensional gels were prepared and run according to the method of O'Farrell (22) and as previously modified (5). Transfer of the proteins to nitrocellulose and immunostaining were carried out as described by Birkett et al. (3).

Fluorescence Microscopy

Myxamebal fluorescence staining was carried out essentially using the method of Havercroft and Gull (11).

Flagellates of the strain LU 352 were prepared according to a protocol devised by Adrian Blindt (University of Leicester). Essentially, the cells were spun down, resuspended in one-fourth of the original volume of growth medium, and then distilled H_2O was added to bring the cells back to their original concentration. After 30 min, cells were centrifuged and resuspended in a one-fourth volume of the diluted medium, and distilled water was added to restore them to their original concentration. After 2 min, the cells were again centrifuged and resuspended in distilled H_2O to the original cell concentration and allowed to flagellate.

Flagellates at a concentration of 10^7 cells/ml were fixed with an equal vol of 5% formaldehyde in distilled water for 8 min. They were then washed once by centrifuging at 600 g for 2 min, resuspending in PBS, and centrifuging again. The cells were resuspended in PBS at a concentration of 10^7 per ml, and permeabilized with an equal vol of 2% Triton X-100 for 5 min. The cells were washed in PBS once and resuspended in 50 μl of primary antibody (dilutions were as follows: 6-11B-1, 1:3; KMX, 1:500; KMP-1, 1:250). They were incubated at room temperature for 45 min. This was followed by washing once in PBS and incubating in 50 μl of fluorescein-labeled goat anti-mouse antibody (Sigma Chemical Co., St. Louis, MO) diluted 1:10 in PBS for 45 min. The flagellates were washed twice by centrifugation and resuspension in PBS. In some preparations, the nuclei of the cells were stained with 4',6-diaminido-2-phenylindole (DAPI)[1] diluted in PBS (to a concentration of 2 μg/ml) by incubating them in this solution for 5 min. This was followed by a final wash in PBS. One drop of a concentrated flagellate suspension was placed on a microscope slide and mounted in an anti-fade mountant (Mowiol/p-phenylene diamine). Flagellates were photographed using a Zeiss epifluorescence microscope.

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1. *Abbreviations used in this paper*: DAPI, 4',6-diaminido-2-phenylindole; MTOC, microtubule-organizing center.
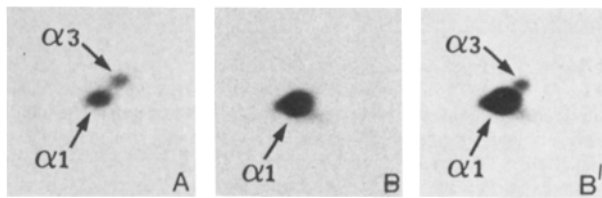
Figure 1. Immunoblotting of *Physarum* CLd flagellate proteins, separated by two-dimensional gel electrophoresis. Only the tubulin areas of the blots are shown. (*A*) Probed with YOL 1/34 showing both the α1 and α3 tubulin isotypes. (*B*) Probed with KMP-1 showing that only the α1 tubulin isotype is detected with this antibody. (*B'*) The same blot as in *B* reprobed with YOL 1/34 to reveal the previously undetected α3 tubulin isotype.

Results

Monoclonal Antibody Specificity

We have made extensive use of two monoclonal antibodies during these studies. The first, 6-11B-1, has been used as the probe for acetylated tubulin in *Physarum*. This antibody (24) was derived from a mouse inoculated with sea urchin sperm axonemes and will only bind with the isotypes of α-tubulin which have been acetylated, either specifically through the action of α-tubulin acetylase in vivo, or nonspecifically by the use of agents such as acetic anhydride. The second antibody that we have used extensively is KMP-1. This antibody was raised against *Physarum polycephalum* myxamebal tubulin. Subsequent characterization showed that this monoclonal antibody recognized the α1 tubulin of myxamebas but did not recognize the plasmodial-specific α2 tubulin, nor indeed the complete family of the plasmodial α1 tubulin isotypes (3). Thus we have already established that KMP-1 can discriminate between α-tubulin isotypes expressed within *Physarum*. On further characterization of KMP-1, we discovered that it was unable to recognize the α3 tubulin isotype that is characteristic of the flagellate. Fig. 1 *A* shows the two α-tubulin isotypes of the *Physarum* flagellate detected by immunoblotting proteins separated on two-dimensional gels with the general anti-α-tubulin monoclonal antibody, YOL 1/34. In contrast, KMP-1 illuminated only a single protein species on the two-dimensional immunoblots. The mobility of the KMP-1-positive spot exactly matched that of α1 tubulin, but to clarify this precise location and also to ensure that the blots contained sufficient amounts of the α3 isotype to register, the same nitrocellulose was subsequently reprobed with YOL 1/34 which clearly demonstrated that α3 tubulin was present but had remained undetected by KMP-1 (Fig. 1, *B* and *B'*). Previous work has shown that the α3 tubulin isotype most probably arises via a posttranslational modification and that the most likely precursor is the α1 tubulin isotype (5, 9). From this evidence it seems that the KMP-1 epitope is significantly altered by changes occurring during the posttranslational modification such that the antibody no longer recognizes its combining site.

The two-dimensional gel coordinates of the *Physarum* α3 tubulin isotype and its relationship to the α1 tubulin are clearly analogous to the pattern of α-tubulins seen in *Chlamydomonas*. In this organism it has been clearly demonstrated that this posttranslational modification involves the acetylation of a single lysine residue of α-tubulin. While the nature of the posttranslational modification of *Physarum*
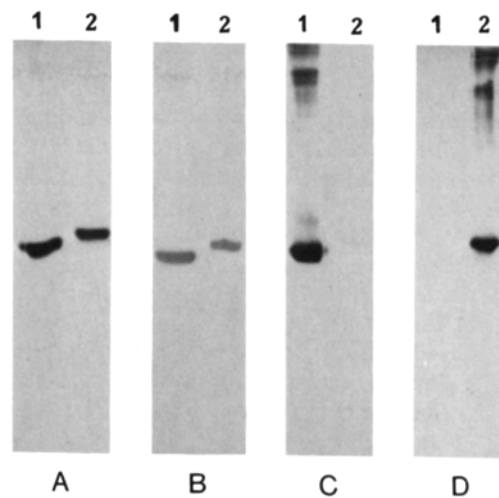

Figure 2. Immunoblotting of chemically acetylated *Physarum* myxamebal tubulin illustrating the selectivity of the KMP-1 and 6-11B-1 antibodies to unacetylated and acetylated tubulin, respectively. Samples were run on one-dimensional gels, blotted onto nitrocellulose, and probed with a series of anti–α-tubulin monoclonal antibodies. Protein was chemically acetylated (lanes 2) or was taken through a mock acetylation using the relevant buffers as a control (lanes *1*). Antibodies used were DM1A, YL 1/2, KMP-1, and 6-11B-1 for *A–D*, respectively.

α-tubulin still remains to be characterized, it is possible that a similar reaction operates. The fact that KMP-1 shows selectivity towards α1 tubulin and does not to bind with α3 tubulin led us to investigate whether the epitope for KMP-1 is susceptible to acetylation and also whether *Physarum* α-tubulin can be recognized by the acetylated-tubulin probe 6-11B-1.

Microtubule protein prepared from myxamebas was acetylated with acetic anhydride using conditions that favor the specific modification of lysine residues. The modification caused the tubulin to migrate more slowly than the unacetylated protein and both the control and modified samples were subject to a certain amount of aggregation. Such cross-linking is a pH-dependent phenomenon which is common to tubulin-containing solutions which are exposed to alkaline pH under nonreducing conditions. Acetylation had little effect upon the binding ability of the antibodies DM1A or YL 1/2 (Fig. 2, *A* and *B*). However, KMP-1 gave a strong reaction with the non-acetylated α-tubulin but barely recognized the acetylated sample (Fig. 2 *C*). Conversely, the epitope recognized by 6-11B-1 was only minimally represented in the myxamebal tubulin before chemical acetylation while the modified α-tubulin band was intensely stained (Fig. 2 *D*). Both KMP-1 and 6-11B-1 stained some of the higher molecular weight material which resulted from the modification procedure but neither antibody identified either β-tubulin or actin (which was present as a minor contaminant from the preparative procedure); it is taken from this that the staining of the higher molecular weight bands is due to the presence of α-tubulin, nonspecifically cross-linked and sufficiently stable to withstand the reducing conditions of standard gel sample preparation.

These results clearly indicate that the 6-11B-1 antibody reacts strongly with acetylated *Physarum* tubulin in a manner similar to previous descriptions of its epitope specificity in other species (24). Furthermore, it appears that recognition
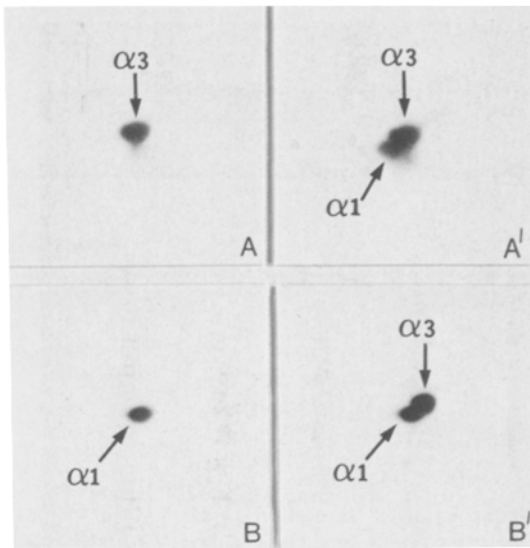
Figure 3. Immunoblotting of LU 352 flagellate proteins separated by two-dimensional gel electrophoresis and probed with the KMP-1 and 6-11B-1 antibodies to show that acetylated tubulin is localized in the α3 tubulin isotype. (*A*) Probed with 6-11B-1. (*A'*) The same blot as in *A* but reprobed with KMP-1. (*B*) Probed with KMP-1. (*B'*) The same blot as in *B* but reprobed with 6-11B-1.

of α-tubulin by KMP-1 can be essentially abolished by acetylation.

Characterization of the Acetylated Tubulin Isotypes of Physarum

Using the 6-11B-1 antibody it was possible to question whether any of the several α-tubulin isotypes in *Physarum* might naturally be subject to acetylation in the organism. We have used a recently developed axenic strain of myxamebas, LU 352, which, unlike many *Physarum* axenic strains, has retained the ability to flagellate. Two-dimensional immunoblots of the proteins of mature LU 352 flagellates were probed with the antibodies 6-11B-1 and KMP-1. Each of these antibodies revealed only a single reaction product, the mobilities of which matched those of the α3 and α1 tubulins, respectively (Fig. 3, *A* and *B*). This specificity of isotype detection was confirmed by reprobing the same blots with the alternative antibody. Reprobing of the 6-11B-1 blot with KMP-1 revealed the previously undetected α1 tubulin isotype (Fig. 3 *A'*) and reprobing the KMP-1 blot with 6-11B-1 revealed the previously undetected α3 tubulin isotype (Fig. 3 *B'*). Therefore acetylated α-tubulin is indeed present in *Physarum* flagellates but is exclusively found as the α3 tubulin isotype.

Investigations involving one-dimensional and two-dimensional gel electrophoresis and even immunoblotting have not previously revealed the bona fide presence of an α3 isotype in any other cell type of the slime mould, but with the availability of the selective and sensitive 6-11B-1 immunological probe, proteins of the myxameba and plasmodium were specifically examined for the presence of acetylated tubulin isotypes. On the two-dimensional immunoblots of the total myxamebal cell proteins, the 6-11B-1 antibody identified a single minor spot, which by its coordinates and subsequent reprobing with KMP-1 proved to be α3 tubulin (Fig. 4, *A* and
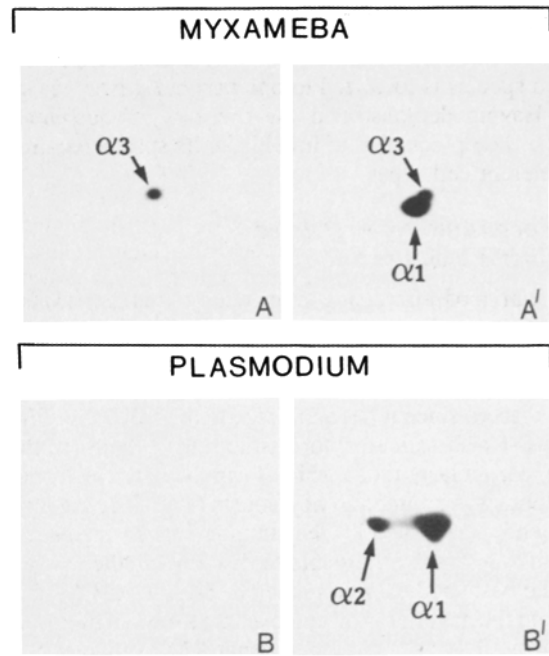
Figure 4. *A* and *A'* are myxamebal proteins separated by two-dimensional gel electrophoresis and immunoblotted with antibodies to reveal the presence of acetylated tubulin in the α3 tubulin isotype. (*A*) Probed with 6-11B-1. (*A'*) The same blot as in *A* but reprobed with KMP-1. (*B*) Purified plasmodial tubulins separated by two-dimensional gel electrophoresis and immunoblotted with 6-11B-1 showing the absence of acetylated tubulin. (*B'*) The same blot as in *B* reprobed with the general anti-α-tubulin antibody DM1A revealing the presence of the plasmodial α-tubulin isotypes.

A'). Similar immunoprobing of purified microtubule protein prepared from CLd AXE myxamebas revealed that even this material contains a very small amount of α3 tubulin (data not shown) which has probably avoided detection on previous occasions because it is completely overshadowed by the abundant α1 tubulin isotype.

Purified microtubule protein from the plasmodial stage of the *Physarum* life cycle (27) was analyzed by two-dimensional gel electrophoresis and electroblotted onto nitrocellulose. Probing these blots with the 6-11B-1 antibody failed to reveal the presence of any acetylated α-tubulin isotypes in this purified preparation. Reprobing the same blots with the anti-α-tubulin antibody DM1A confirmed that the full complement of plasmodial α-tubulin isotypes were indeed present (Fig. 4, *B* and *B'*). This purified microtubule protein was derived from a microplasmodial culture which contains both interphase and mitotic microplasmodia. In the *Physarum* plasmodium, tubulin is only assembled in the mitotic spindle microtubules during the synchronous mitosis. To maximize the possibility of detecting acetylated forms of α-tubulin which may only be present at times of microtubule polymerization, we also blotted samples taken from single macroplasmodia in both interphase and mitotic periods. Again, on probing these preparations with the 6-11B-1 antibody, no acetylated α-tubulin was detected.

Therefore, immunoblotting conducted with the different cell types of *Physarum* has revealed that acetylated α-tubulin is present in the flagellate where it can constitute a major α-tubulin species, and in the myxameba where it is a rela-

tively minor isotype, but seemingly not at all in the plasmodium. In both of the cell types where it is found, the acetylated species is localized to one particular isotype, α3 tubulin. Having demonstrated the presence of acetylated tubulin we then proceeded to investigate its spatial location in the different cell types.

Spatial Localization of Acetylated α-Tubulin in Physarum

Indirect immunofluorescence microscopy was performed with the 6-11B-1 antibody on mature flagellate cells. The primary flagellum was brightly stained in all cells. The shorter secondary flagellum was also stained but was generally harder to discern since it lies adpressed to the cell body (Fig. 5 A). 6-11B-1 also stained cytoplasmic microtubules in the flagellate, particularly those which form the anterior "cone" of microtubules surrounding the nucleus (Fig. 5 A; see also Fig. 8 D and D'). Generally the staining was so intense in this region that it was impossible to discern whether or not the flagellar basal bodies were stained by 6-11B-1. KMP-1 also stained both the flagellum and cytoplasmic arrays of microtubules (Fig. 5, B and C). It appears that KMP-1 stained the flagellum less well than 6-11B-1 but stained more microtubules within the posterior half of the flagellate; however, such subtle differences require further examination before being seen as significant. The microtubule-containing structures visualized by both antibodies represents the complete range which the flagellate can assemble; being a nondividing cell type, spindle microtubules are not produced.

The overall arrangement of cytoplasmic microtubules in an interphase myxameba was revealed by immunofluorescence microscopy using an anti-β-tubulin monoclonal antibody KMX-1 (Fig. 6 A). Use of the KMP-1 antibody revealed a similar arrangement of cytoplasmic microtubules (Fig. 6 B). The 6-11B-1 staining pattern of acetylated tubulin was dramatically different. The extensive network of cytoplasmic microtubules was not stained by the 6-11B-1 antibody but acetylated tubulin was solely located in two distinct, small structures close to the nucleus (Fig. 6, C and C'). If the cells lay in a favorable focal plane, high resolution micrographs indicated that the acetylated tubulin–containing bodies were short rod-shaped structures, often orthogonally opposed to one another (Fig. 6, D–G). This and other features are characteristics of the centrioles in this Physarum cell type (8, 12, 32). In some cells, there was an indication that one of the very short microtubular arrays adpressed to the centriole was also stained. Mitotic myxamebas visualized with KMP-1 showed a distinct spindle apparatus (Fig. 7 A). In contrast, the 6-11B-1 antibody gave no staining of the spindle, thus indicating that the microtubules of the mitotic spindle were totally devoid of acetylated tubulin throughout mitosis (Fig. 7 B). However, acetylated tubulin was still present in small structures which lay at the poles of the spindle during mitosis (Fig. 7, B and B'). The characteristic positioning and shape of these bodies in the interphase cell and the manner in which they split and migrate to reside at the spindle poles for the duration of mitosis (8, 12, 32) leads us to suggest that it is indeed the microtubules of the centrioles which contain acetylated tubulin.

Immunofluorescence microscopy of smears taken from a Physarum macroplasmodium at interphase and mitosis and stained with the 6-11B-1 antibody showed no acetylated
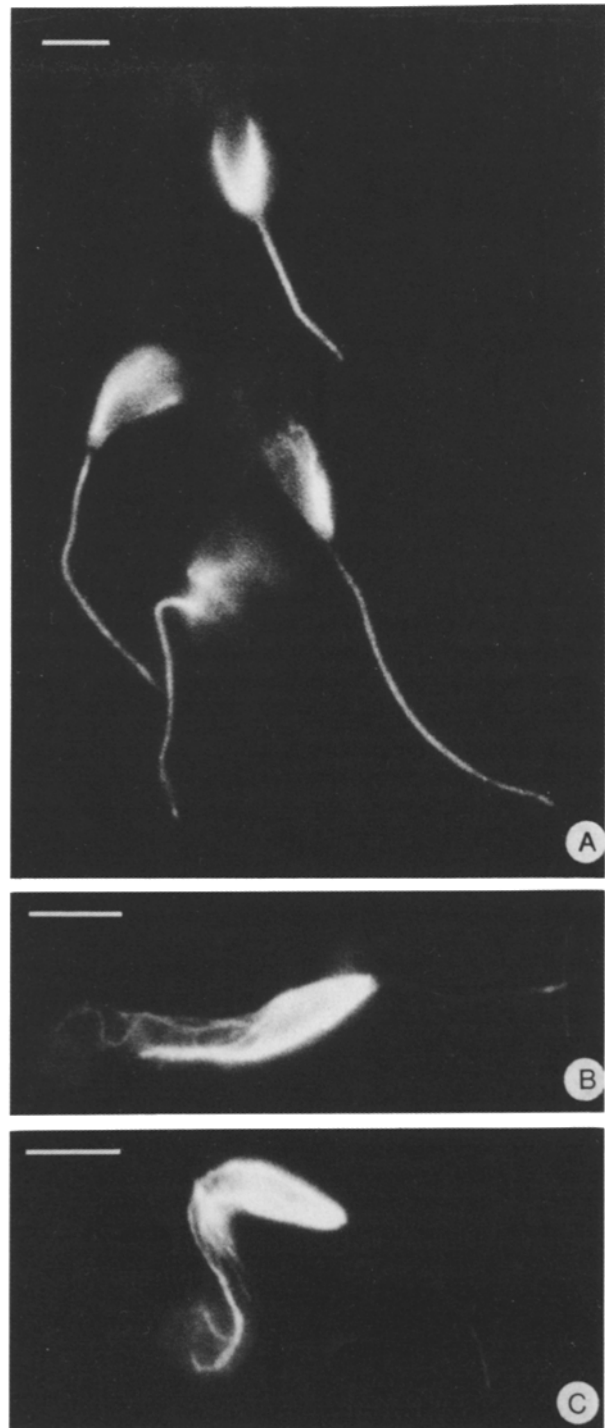
Figure 5. (A) Immunofluorescence detection of acetylated α-tubulin in the flagella and cell body cone microtubules of flagellates by staining with the 6-11B-1 antibody. (B and C) Immunofluorescence detection of unacetylated α-tubulin in the flagellates by staining with the KMP-1 antibody. There is some staining of the flagellum although the cone, and cytoplasmic microtubules extending into the posterior of the cell are well stained. Bar, 5 μm.

microtubule structures, thus confirming the earlier immunoblotting experiments indicating that the plasmodial cell type does not possess acetylated tubulin.
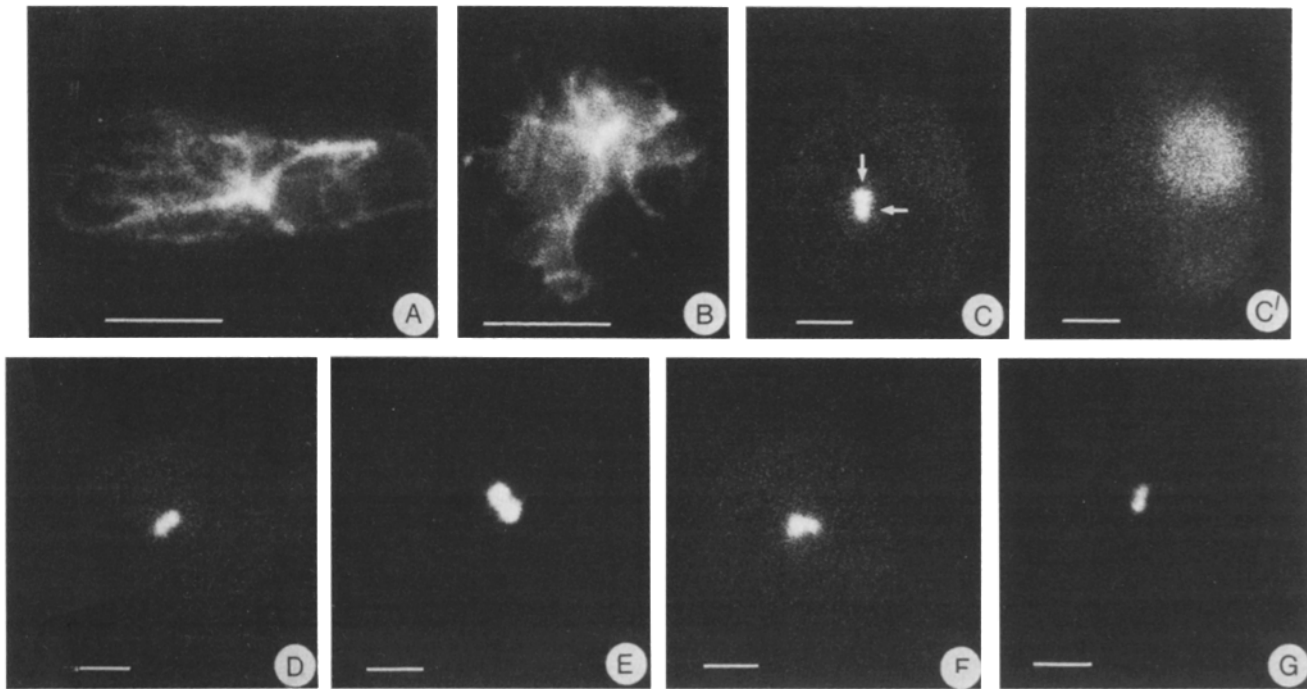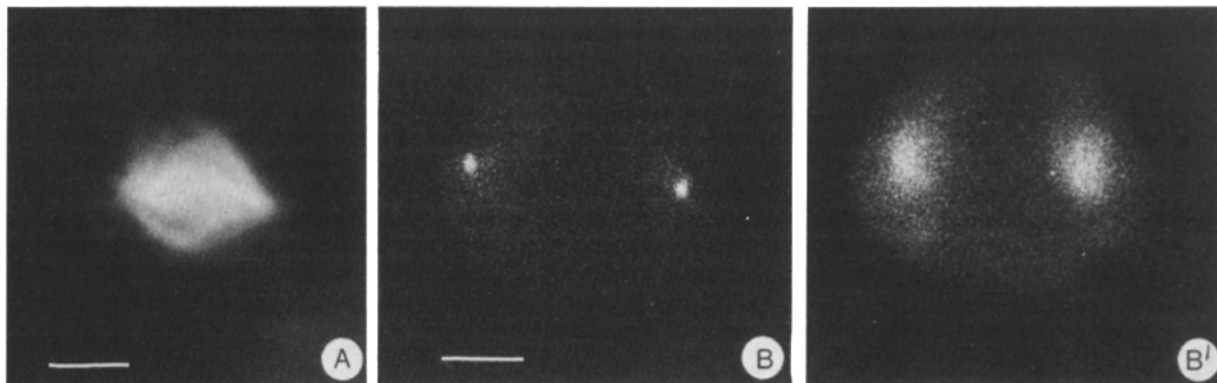
Figure 6. Immunofluorescence staining of the interphase array of microtubules in a myxamebal cell using (*A*) the anti-β-tubulin monoclonal KMX-1, and (*B*) the KMP-1 antibody. (*C*) Immunofluorescence staining of a myxamebal cell using the 6-11B-1 antibody. Staining is localized to the two centrioles in this cell type. *C'* is the same cell as in *C* but stained with DAPI to reveal the nucleus. *D–G* are other examples of myxamebas stained with the 6-11B-1 antibody to reveal the presence of acetylated α-tubulin in the centrioles but not the cytoplasmic microtubules. Bars: (*A* and *B*) 5 μm; (*C–G*) 1 μm.

The Elaboration of Acetylated Microtubules during the Myxamebal-Flagellate Transformation

Physarum myxamebas can transform into flagellates and this transformation is accompanied by an extensive remodeling of cellular organization. Over a 60–90-min period, myxamebas lose their rounded shape, becoming elongate cells with the nucleus at the anterior end and also developing one short and one long flagellum (23).
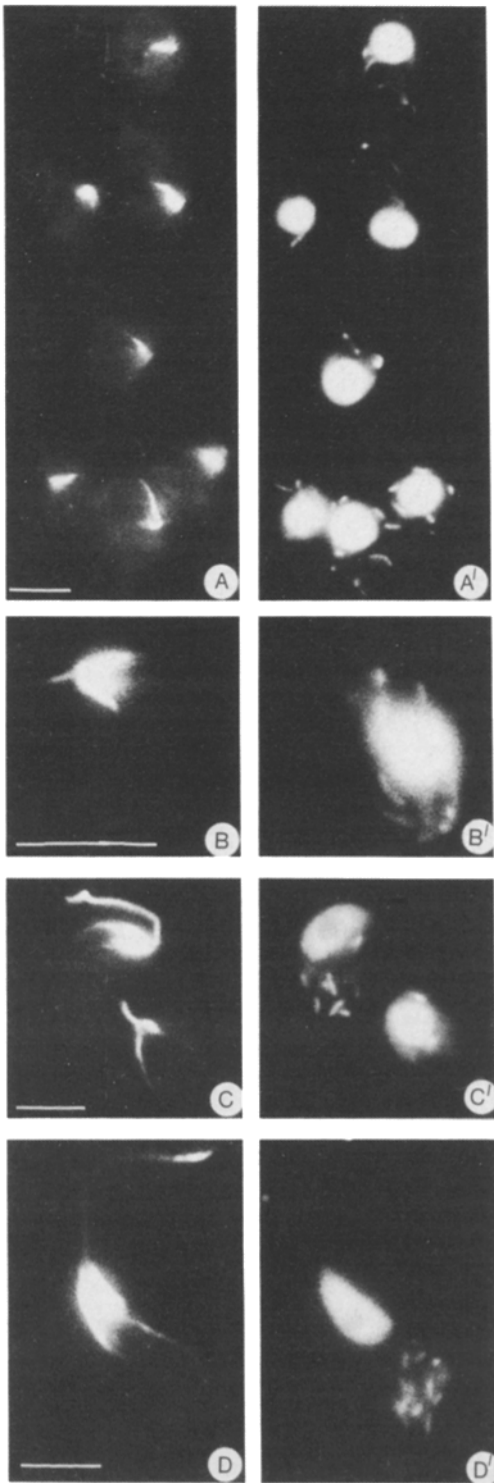
We have used the 6-11B-1 antibody to follow changes in the acetylated microtubules during the myxamebal–flagellate transformation. Early in the transformation a small group of acetylated microtubules become apparent. They are located close to the original myxamebal centrioles and often assume a spur-like morphology (Fig. 8, *A* and *A'*). At this stage the cells have not lost their overall ameboid appearance. Mitochondria stained by the DNA intercalating dye DAPI are distributed apparently randomly around the cell nucleus (Fig. 8 *A'*). The complement of acetylated microtubules has increased dramatically in the anterior region of the cell by the time that the primary flagellum emerges from the cell (Fig. 8, *B* and *C*). At a later stage of the transformation, the now elongate cell contains an extensive cone of acetylated microtubules together with some that extend further into the cell body. In such elongated cells, the nucleus has adopted its anterior position and the mitochondria are located in the posterior portion of the cell (Fig. 8, *D* and *D'*).

Figure 7. Immunofluorescence microscopy of mitotic myxamebas showing (*A*) unacetylated microtubules in the spindle stained with KMP-1 and (*B*) acetylated α-tubulin stained with 6-11B-1 in structures at the poles of the spindle. *B'* is a DAPI-stained image of the same cell as in *B* to show the position of the late telophase chromosomes. Bar, 2 μm.

Discussion

Acetylated Tubulin Isotypes and Antibody Recognition Epitopes

The results of our chemical acetylation studies show that the two antibodies that we have used extensively in this study possess differential reactivities dependent upon the presence or absence of acetylated lysine residues within the α-tubulin polypeptide. We have been able to show that the 6-11B-1 antibody reacts with acetylated *Physarum* α-tubulin in a manner similar to that reported in the initial description of this antibody with other α-tubulins (24). The KMP-1 antibody shows an opposite selectivity in that it fails to recognize acetylated *Physarum* α-tubulin. Thus, these two antibodies, 6-11B-1 and KMP-1, provide operational probes for acetylated and un-acetylated *Physarum* α-tubulin, respectively.

Concomitant use of these two antibodies has allowed us to search for the presence of naturally acetylated α-tubulins that are expressed in the *Physarum* life cycle. Using this approach we have successfully demonstrated the presence of this posttranslationally modified tubulin in both flagellates and myxamebas. In both cases, the acetylated α-tubulin exists as the α3 isotype. We can find no evidence for the presence of acetylated tubulin, nor the α3 tubulin isotype, in the plasmodial stage of the *Physarum* life cycle. Previous work has demonstrated that the α3 isotype is generated from the α1 isotype via a posttranslational modification (5, 9). Our present results now show that this posttranslational modification involves an acetylation reaction. Of course, it should be noted that other cryptic modifications may also occur during the transformation from the α1 isotype to the α3 isotype.

The data presented previously for the 6-11B-1 antibody (24) and confirmed here with *Physarum* tubulin suggest that the epitope might actually span the particular lysine residue of α-tubulin at which α-tubulin acetylase acts. Similarly, the KMP-1 epitope appears also to include the lysine residue which is the target for the α-tubulin acetylase: KMP-1 recognizing this region of the unacetylated α-tubulin polypeptide and 6-11B-1 recognizing the region when the lysine is acetylated. Although this appears the most likely explanation, it is, of course, possible that for each antibody the epitope might instead be merely created or destroyed at a site away from the actual site of acetylation by a process that is nevertheless entirely dependent upon the modification; for example, by an acetylation-induced conformational change. However, evaluation of all the data, particularly the chemical acetylation results, suggests that this is the more unlikely explanation. Consideration of other information suggests that the KMP-1 and 6-11B-1 recognition epitopes are in fact not merely the same region modulated by the presence or absence of an acetyl group on the included lysine. This information comes from the knowledge that the 6-11B-1 antibody reacts with acetylated α-tubulin from an evolutionarily diverse group of organisms. However, the KMP-1 antibody appears to be very selective for slime mould tubulin and does not react with the α-tubulins of a great many fungi, algae, protozoa, higher plants, birds, insects, or mammals (2, 3).

Figure 8. (*Left*) Immunofluorescence detection of the elaboration of acetylated microtubules during the myxamebal–flagellate transformation. (*Right*) The corresponding DAPI images of the cells. In the right-column micrographs, both the nucleus and mitochondria of each cell can be seen by detection of DNA using the DAPI staining. (*A*) Initially a spur of acetylated microtubules develop from the centriole region. (*B*) A cell showing acetylated microtubules in the cone and in the short, forming flagellum. The mitochondria are still randomly orientated around the cell nucleus. (*C*) Cells at slightly later stages in the transformation. (*D*) A mature flagellate can be seen in this micrograph showing acetylated tubulin in the flagellum, in the microtubules of the anterior cell cone, and in microtubules extending back into the posterior cell body. In this cell the mitochondria are localized in a discrete group in the posterior region of the cell. Bar, 5 μm.

The slight difference in epitope between the two antibodies is of some interest in the context of the other α-tubulin isotypes expressed in the *Physarum* life cycle. Previously, we have shown that the KMP-1 antibody does not react with the plasmodial-specific α2 tubulin isotype nor a subset of the plasmodial α1 tubulin isotypes (3). Since we have now demonstrated that the 6-11B-1 antibody does not recognize any of the plasmodial α1 or α2 tubulin isotypes, then the lack of KMP-1 reactivity of the plasmodial α2 and certain α1 tubulin isotypes cannot be due to the fact that they are acetylated. Rather, it appears likely that these plasmodial α-tubulin isotypes possess structural alterations in the polypeptide at, or close to, the lysine acetylation site. If this alteration is a change in the primary structure of the polypeptide in this region, then this information will be pertinent to the isotype identification of cloned tubulin genes as they become available.

Cellular Localization and Stable Microtubules

Even though our immunoblotting studies show that both the myxamebal and flagellate cells possess the acetylated α3 tubulin isotype, there is an intriguing difference in its cellular location between these two cell types. In the flagellate, acetylated tubulin is present in both the flagellum axonemes and in cytoplasmic microtubules in the cell body. The presence of acetylated tubulin in the *Physarum* flagellum axonemes fits well with the original description of this isotype in *Chlamydomonas* (15, 16, 20) and the more recent descriptions of other species using the 6-11B-1 antibody (24). Our observations on the *Physarum* flagellate clearly show that acetylated tubulin is not confined to the axoneme. Indeed a very extensive array of acetylated microtubules is located in the cell body. Recently, a subset of acetylated cytoplasmic microtubules has also been identified in the *Chlamydomonas* cell body (14). *Physarum* myxamebas exhibit a unique localization of acetylated tubulin to certain microtubule structures within the cell. Its location in or near the centrioles and not in the abundant network of cytoplasmic microtubules in interphase nor in the spindle microtubules during mitosis suggests a complex level of control over the activity of the α-tubulin acetylase enzyme or the distribution of acetylated tubulin within one cellular compartment.

There is, as yet, no direct explanation for the function of the acetylated tubulin isotype. Our results show that in *Physarum* it is present in or associated with microtubules of different polymeric form; i.e., the triplet microtubules of the centrioles, the doublet microtubules of the axoneme, and the singlet microtubules of the flagellate cytoplasm. This suggests that it does not functionally define a particular polymeric form of microtubule construction. However, the microtubule structures that do possess acetylated α-tubulin do have certain common characteristics. In particular, they are all rather stable, cross-linked microtubule organelles (12, 23, 31, 32). The centriole and flagellum axonemes are acknowledged to be stable, cross-linked structures. The microtubules of the flagellate cone have been shown to exist in highly ordered arrays, to be cross-linked, stable to relatively harsh extraction conditions, and indeed may be responsible for the elongated shape of the flagellate in comparison to the myxameba (23, 31). In contrast, those microtubule structures that appear to lack the acetylated isotype, the individual myxamebal cytoplasmic and mitotic microtubules

and the plasmodial intranuclear mitotic spindle microtubules, do not represent a population of stably cross-linked microtubules. Also, although one cannot make statements regarding the mean residence time of individual α-tubulin molecules within, say, the myxamebal cytoplasmic microtubules, it is clear that the microtubules of the myxamebal and plasmodial spindles are ephemeral. Thus, it is apparent that there is a correlation between the possession of acetylated tubulin and the natural stability of particular microtubular organelles. There is, of course, no proof of a direct causal link between the acetylated α-tubulin and the production of stable microtubules. In fact, we have previously found no markedly unusual characteristics of acetylated tubulin during assembly/disassembly in vitro (29). However, the true in vivo role of acetylated tubulin is likely to involve the participation of other proteins and in this context it is intriguing that acetylated α-tubulin is associated with cross-linked microtubular arrays and that these arrays are not absolutely associated with one particular cellular function.

Control of Selective Acetylation

The elaboration of acetylated microtubules during the myxameba–flagellate transformation raises the question of how microtubules acquire the acetylated α-tubulin. Is there an absolute link between microtubule assembly and acetylation of α-tubulin, such that these flagellate cytoplasmic microtubules represent new microtubules polymerized during the transformation? Or, can α-tubulin molecules that are already present in microtubules become acetylated? Suggesting that at least some of the acetylated flagellate cytoplasmic microtubules may represent microtubules that existed in the myxamebal cytoplasm but were acetylated subsequently, during the transformation to a flagellate. We can, at present, provide little evidence that addresses these questions directly and feel that immunolocalization of α-tubulin at the electron microscope level will be informative. However, it is pertinent to note that the microtubule arrays of both the myxameba and the flagellate are precisely ordered structures and that during the transformation, well-described groups of microtubules are polymerized (12, 23, 31). Further, it appears that many of these microtubule arrays may be nucleated by microtubule-organizing centers (MTOCs) that are different to the main MTOC (MTOC 1) that nucleates the major network of myxamebal interphase cytoplasmic and mitotic spindle microtubules (8, 12, 31, 32). Thus, it may be that in *Physarum* and other eukaryotic cells there are at least two types of MTOCs operating: acetylating MTOCs and non-acetylating MTOCs. If control of acetylation is initially exercised by particular MTOCs, then presumably a mechanism also exists to subsequently maintain acetylation control at a point of microtubule assembly distal to the nucleating MTOC. Conversely, acetylation may be independent of the initial influence of particular MTOCs but may be more related to the nature of the microtubule array produced, the kinetics of its assembly/turnover, or other events in its subsequent history. It appears clear that in order to distinguish between these various levels of control we will need to learn more about the relationship between the kinetics of acetylation and microtubule construction. In this respect, the cells of many eukaryotic microbes with their highly ordered and diverse microtubule arrays should provide valuable experimental models.

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