Distribution of Acetylated Alpha-Tubulin in *Physarum polycephalum*

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Abstract. The expression and cytological distribution of acetylated alpha-tubulin was investigated in *Physarum polycephalum*. A monoclonal antibody specific for acetylated alpha-tubulin, 6-11B-1 (Piperno, G., and M. T. Fuller, 1985, *J. Cell Biol.*, 101:2085–2094), was used to screen for this protein during three different stages of the *Physarum* life cycle—the amoeba, the flagellate, and the plasmodium. Western blots of two-dimensional gels of amoebal and flagellate proteins reveal that this antibody recognizes the α3 tubulin isotype, which was previously shown to be formed by posttranslational modification (Green, L. L., and W. F. Dove, 1984, *Mol. Cell. Biol.*, 4:1706–1711). Double-label immunofluorescence demonstrates that, in the flagellate, acetylated alpha-tubulin is localized in the flagella and flagellar cone. Similar experiments with amoebae interestingly reveal that only within the microtubule organizing center (MTOC) are there detectable amounts of acetylated alpha-tubulin. In contrast, the plasmodial stage gives no evidence for acetylated alpha-tubulin by Western blotting or by immunofluorescence.

MICROTUBULES, an important component of most eukaryotic cells, participate in mitosis, cytoskeletal organization, intracellular transport, and flagellar and ciliary movement. The primary component of microtubules is the heterodimer of alpha- and beta-tubulin. The different functions associated with microtubules could be realized by changes in tubulin primary structure, either by the use of different tubulin gene products or by posttranslational modification of these proteins. Distinct tubulin gene products may be associated with distinct microtubule functions (18). Many organisms possess multigene tubulin families (6). Tubulin also may be altered functionally as a result of posttranslational modifications (14). Several posttranslational modifications are known to occur; phosphorylation of beta-tubulin (9), tyrosination of alpha-tubulin (2), and acetylation of alpha-tubulin have been documented (16). These mechanisms are not mutually exclusive; multiple possibilities may exist in concert.

Acetylated alpha-tubulin, first discovered by L'Hernault and Rosenbaum (16), is one form of posttranslational modification that may be important in determining microtubule organization and function. Piperno and Fuller (19) described a monoclonal antibody, 6-11B-1, that is specific for acetylated alpha-tubulin. The two lines of evidence for the specificity of this monoclonal antibody are, first, the creation of an epitope by in vitro acetylation of alpha-tubulin and, second, the recognition of *Chlamydomonas* axoneme alpha-tubulin that is known to be acetylated (16, 19). This antigen is restricted to certain microtubular structures. Immunoblots have shown that 6-11B-1 recognizes a subset of the alpha-tubulin from the flagella of *Chlamydomonas*, *Drosophila* sperm, human sperm, and the cilia of sea urchin blastulae (19). Immunofluorescence assays have also shown that acetylated alpha-tubulin is present in the basal bodies and a subset of the cytoplasmic microtubules in *Chlamydomonas* (14). These cytoplasmic microtubules containing acetylated alpha-tubulin exhibit biochemically distinct properties; compared with microtubules that lack acetylated alpha-tubulin, they are more resistant to nocodazole or colchicine treatment, although they show similar sensitivity to 0°C (14).

The many developmental stages of *Physarum* (21) make this a useful organism in which to study acetylated alpha-tubulin. *Physarum* has two proliferative stages, the amoebal and the plasmodial. Uninucleate amoebae differentiate into multinucleate plasmodia in two different ways: either a pair of amoebae mate, or a single amoeba of a certain strain undergoes apogamic development. The plasmodium carries out rounds of nuclear division without cytokinesis. Upon starvation and exposure to light, the plasmodium undergoes meiosis and produces spores. These spores hatch into amoebae. Most strains of amoebae, when suspended in water, reversibly form flagellates, a nonproliferative cell type.

The tubulin proteins and microtubular organization during the life cycle of *Physarum* have been analyzed (20, 23). Amoebal microtubular structures include the mitotic spindle, the centrioles, and the cytoskeleton (11, 12, 15, 27). The uninucleate amoeba has an open mitosis in which the cytoplasmic microtubule organizing centers (MTOCs)[1] serve as the two mitotic poles. The nonproliferative flagellate contains both a long and a short flagellum, as well as a supporting structure called the flagellar cone, composed of two basal

1. *Abbreviation used in this paper*: MTOC(s), microtubule organizing center(s).

bodies and a complex array of microtubules (28). In contrast, the plasmodium is a syncytium containing up to 10^9 nuclei that synchronously progress through the cell cycle. The only microtubular structure detected in the plasmodium is the mitotic spindle (11). Intranuclear MTOCs, which do not contain centrioles, are used during the closed plasmodial mitosis (25).

Distinct sets of tubulins are found in the amoebal and plasmodial stages. From the amoebae only one beta-tubulin and one alpha-tubulin can be resolved in two-dimensional gels (4). Analysis of mutants, however, reveals that two gene products encode the amoebal β1-tubulin isoform (5). Previous work from our laboratory has shown that during flagellate formation a novel alpha-tubulin, α3, is made by post-translational modification of a preexisting polypeptide, most likely α1 tubulin (10); α3-tubulin is more acidic and of slightly slower electrophoretic mobility than α1. From the plasmodium, two alpha-tubulins, α1 and α2, and two beta tubulins, β1 and β2, can be resolved in two-dimensional gels (4). One-dimensional isoelectric focusing gels reveal that α1 can be resolved into several subspecies (3).

The following study was prompted by several questions: (*a*) Is acetylation one of the posttranslational modifications that occur during flagellate formation in *Physarum*? The more acidic isoelectric point of α3-tubulin is consistent with such a change. In *Chlamydomonas*, acetylation of alpha-tubulin has been shown to occur during flagellate formation (16). (*b*) Do the amoeba and the plasmodium contain this modified form of alpha-tubulin? (*c*) If alpha-tubulin is being acetylated, is it localized in specific subcellular structures in *Physarum*? In this study we have used the 6-11B-1 antibody for Western blotting and indirect immunofluorescence staining experiments to examine the expression and cytological distribution of acetylated alpha-tubulin.

Materials and Methods

Strains and Culture Conditions

CLd-AXE (17) myxamoebae were cultured in MYM + hemin, a complex mycological medium (22), in shaker flasks at 26°C, 150 rpm, and used at a concentration of 4–8 × 10^6/ml. Nonaxenic CLd (7) were grown on an *Escherichia coli* bacterial lawn on SM (1) plates to a concentration of 8 × 10^6 per 9 cm plate.

LU215 (5) × CLd plasmodia were maintained as microplasmodia in MYM, shaken at 150 rpm, at 26°C. When 100 ml of culture reached 30–50 mg/ml, the plasmodia were pelleted, resuspended in 1/2 pellet volume of medium, and inoculated onto two filters (No. 576 filter paper, Schleicher & Schuell, Inc., Keene, NH). Once the excess medium had evaporated, 150 ml of medium was added.

In Vivo Labeling of Polypeptides

Axenic amoebae were labeled by adding 1 mCi of [^{35}S]methionine (Amersham Corp., Arlington Heights, IL) to 5 ml of the medium. Nonaxenic amoebae were labeled while growing on bacteria in the presence of 1 mCi of [^{35}S]methionine in a basal salts solution (24). Plasmodia were labeled ~1 h before the second synchronous mitosis by transferring half of a plasmodium to a 14-cm petri dish containing 2 ml of MYM medium and 2 mCi of [^{35}S]methionine. The unlabeled half of the plasmodium was monitored by immunofluorescence to judge the stage of mitosis and synchrony.

Induction of Flagellate Formation

Plate-grown CLd were washed off with 10 ml of deionized water/plate. The resulting suspension was centrifuged for 2.5 min at 1,000 rpm in a Damon/IEC centrifuge (International Equipment Co., Needham Heights, MA). The amoebal pellet was resuspended in 10–20 ml deionized water and shaken 1–2 h at 26°C, ~150 rpm. Flagellate formation occurred in ~75% of the amoebae.

Western Blotting

Two-dimensional gels were run as described by Burland et al. (4) except that a 1:3 ratio of 3.5–10:5–7 ampholines was used for the amoebal and flagellate samples. The transfer of proteins to nitrocellulose was done according to the method of Towbin (26) with the following modifications. Proteins were transferred to nitrocellulose with a pore size of 0.2 μm (Schleicher & Schuell) in 20 mM Tris:150 mM glycine:20% methanol transfer buffer. The electrode separation was 6.5 cm. The transfer ran 12 h at 100 mA. Immunoblotting was done as described by Birkett et al. (3). The primary antibodies used are described below. The secondary antibodies were affinity-purified peroxidase-conjugated goat anti-mouse IgG and IgM (Zymed Laboratories, Inc., San Francisco, CA). Blots were exposed to XAR film for 1 wk at −80°C.

Immunofluorescence

The following antibodies were used. The antibody 6-11B-1 (19) is a monoclonal antibody to acetylated alpha-tubulin, raised against sea urchin sperm. This IgG antibody was obtained in serum-free supernatant fluid and used undiluted (gift from G. Piperno, The Rockefeller University). KMP-1 (3) is a monoclonal antibody to alpha-tubulin, raised against *Physarum* tubulin. This IgM antibody was used at a 1/500 dilution of ascites fluid. KMX-1 (3) is a monoclonal antibody to beta-tubulin, raised against *Physarum* tubulin. This IgG2b antibody was used at a 1/1,000 dilution of ascites fluid. A fluorescein-conjugated sheep anti-mouse secondary antibody against IgG was used to detect the 6-11B-1 staining pattern (Cappel Laboratories, Inc., Malvern, PA). A Texas Red-labeled affinity purified goat anti-mouse secondary antibody against IgM was used to detect the KMP-1 immune complexes (Southern Biotechnology Associates, Birmingham, AL).

KMX-1 was used as a positive control for all Western blot and single-label immunofluorescence experiments because it is a mouse IgG, as is 6-11B-1. KMP-1 was used in the double-label immunofluorescence experiments because it is a mouse IgM. To complement the amoebal and flagellate double-label experiments, the amoebal and flagellate blots were also probed with KMP-1. The KMP-1-probed Western blots are depicted in Fig. 1 because of the interesting decrease of KMP-1's reactivity with α3.

Plasmodial single-label immunofluorescence was done as previously described (11). KMX-1 and 6-11B-1 were used to probe the plasmodial samples. Amoebal double-label immunofluorescent samples were prepared by fixing 2–4 × 10^7 amoebae for 10 min in 3.7% EM grade formaldehyde (Ladd Research Industries, Inc., Burlington, VT), 0.025% Nonidet P-40 (Sigma Chemical Co., St. Louis, MO) in PBS (8). Fixed amoebae were pelleted and resuspended in 1–2 ml of PBS. The amoebae were then allowed to settle onto poly-L-lysine (1 mg/ml) (Sigma Chemical Co.) coated coverslips (1.5, 18 mm^2; Corning Glass Works, Corning, NY) for 1 h. All subsequent washes and incubations were done in 2.5% FBS (Gibco, Grand Island, NY) in PBS. The sequence of steps was: wash; 6-11B-1 incubation; wash; anti-IgG secondary antibody incubation; wash; KMP-1 incubation; wash; anti-IgM secondary antibody incubation; wash; distilled water rinse. Each series of washes consisted of three rinses in FBS-PBS for 10 min each. Primary incubations involved adding 50 μl of antibody for 1 h at 37°C in a humid chamber. Secondary incubations involved adding 50 μl of antibody for 0.5 h at 26°C. The slides were preserved in nonbleaching mounting fluid, which was a generous gift from G. Borisy (University of Wisconsin, Madison, WI) (Sammak, P. J., and G. B. Borisy, manuscript in preparation). Slides were stored at −20°C until observation with a Zeiss Universal microscope with 100× Planapo lens.

Photography

Immunofluorescence slides were recorded on Tri-X (400 ASA) for amoebal and plasmodial slides and Tech Pan (200 ASA) for flagellate slides (Eastman Kodak Co., Rochester, NY). The developed black and white film was printed on FC3 paper (AGFA-GEVAERT, Germany).

Results

An Antibody Specific for Acetylated Alpha-Tubulin Reacts with an Alpha-Tubulin in Flagellates and Amoebae

To determine whether the posttranslational modification of alpha-tubulin that occurs during flagellate formation includes acetylation, [^{35}S]methionine-labeled flagellate poly-

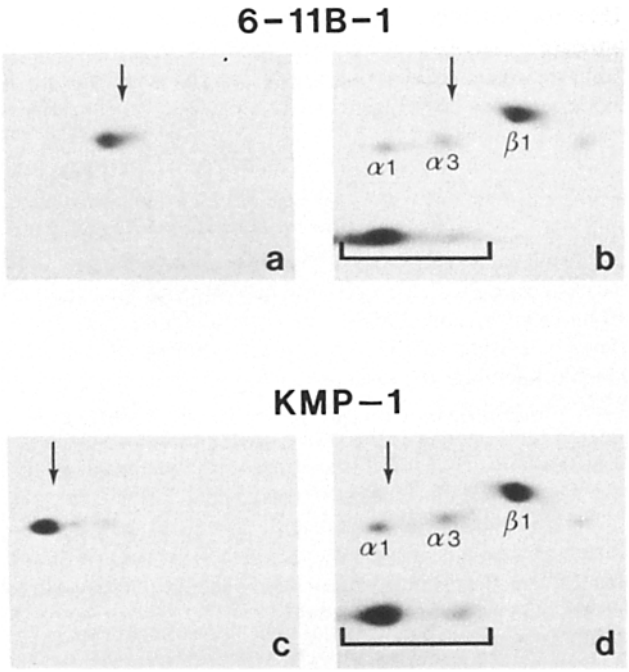


Figure 1. Western blots and the corresponding autoradiographs of ^{35}S-labeled flagellate samples, comparing the 6-11B-1 and the KMP-1 staining patterns. (*a*) 6-11B-1 probed Western blot; (*b*) autoradiograph of the Western blot in *a*; (*c*) KMP-1 probed Western blot; (*d*) autoradiograph of KMP-1 probed Western blot in *c*. 6-11B-1 reacts with α3; KMP-1 reacts more strongly with α1 than with α3. The bar labels the actin position in *b* and *d*. The arrows mark the radioactive ink spotted on the blots to align the blots and autoradiographs.

peptides were electrophoresed on a two-dimensional gel and examined by immunoblotting. These immunoblots were then exposed to film to obtain the total protein pattern and identify the reacting species. Three identical gels were run and blotted simultaneously. The first blot was incubated with 6-11B-1, to detect acetylated alpha-tubulin; the second, with KMP-1, to detect alpha-tubulin; and the third, with only secondary antibody, to detect nonspecific binding. Fig. 1 presents the blots probed with 6-11B-1 (*a*) and KMP-1 (*c*), along with their corresponding autoradiographs (*b* and *d*, respectively). Superimposing the autoradiograph and the blot reveals that the 6-11B-1 recognizes α3-tubulin exclusively, while KMP-1 reacts strongly with α1 but weakly, if at all, with α3. Secondary antibody did not bind in the absence of primary antibody (data not shown).

To determine the reaction of 6-11B-1 with amoebal tubulin it was necessary to harvest amoebae under conditions that would not induce flagellate formation and the production of α3-tubulin. Two different methods were used to obtain nonflagellate samples. First, plate-grown CLd amoebae were harvested at 0°C, which slows down flagellate formation. Second, an axenic strain of amoebae was grown in axenic medium, which inhibits flagellate formation. Western blot analysis of amoebal proteins shows that the trace level of reaction of the antibody 6-11B-1 corresponds to the α3 position (data not shown). There are two possible explanations for observing acetylated alpha-tubulin in growing amoebae. Perhaps the shock of harvesting the "nonflagellated" samples may have induced acetylation of alpha-tubulin, or perhaps small amounts of acetylated alpha-tubulin may be present in all

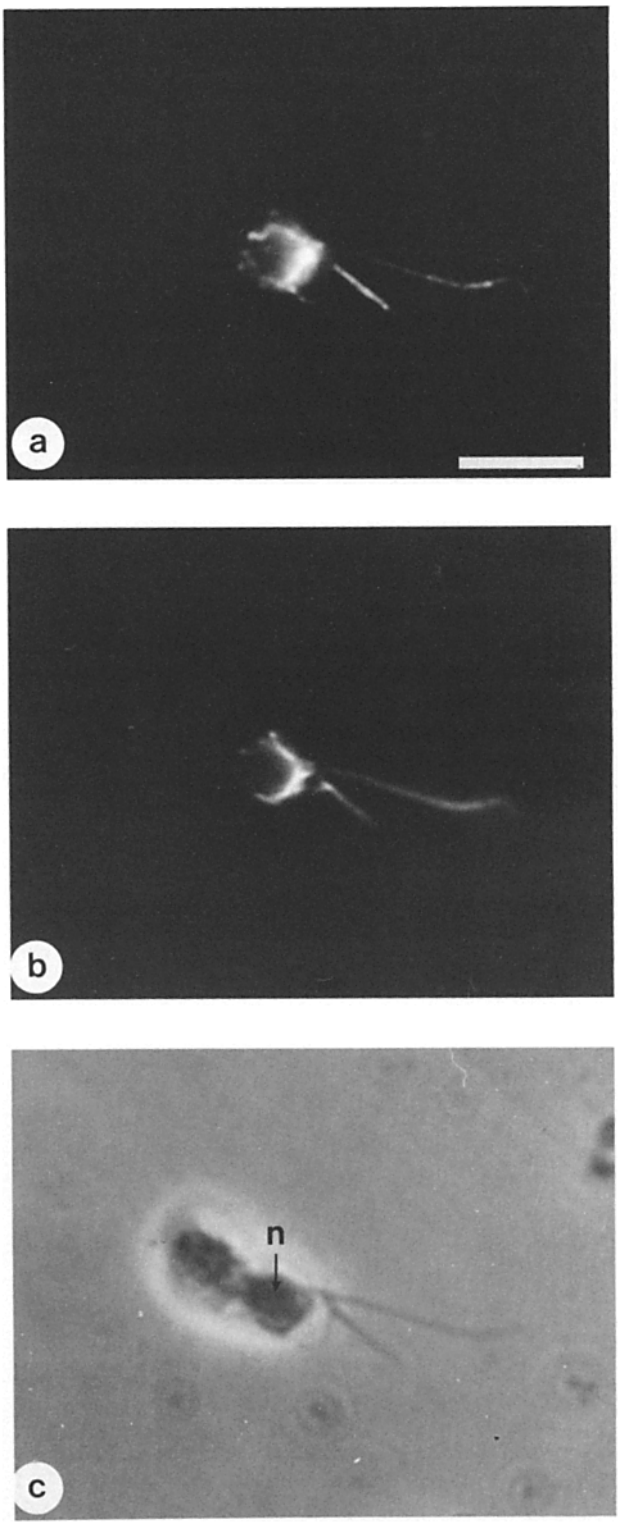


Figure 2. Flagellates stained by double-label indirect immunofluorescence with KMP-1 and 6-11B-1. (*a*) Fluorescence photomicrograph of KMP-1-Texas Red staining pattern shows KMP-1 recognizes the long and short flagella and the flagellar cone; (*b*) fluorescence photomicrograph of 6-11B-1-fluorescein staining pattern shows 6-11B-1 also recognizes both flagella and the flagellar cone; (*c*) phase contrast photomicrograph shows the flagella and cone are near the nucleus (*n*). Bar, 2 μm.

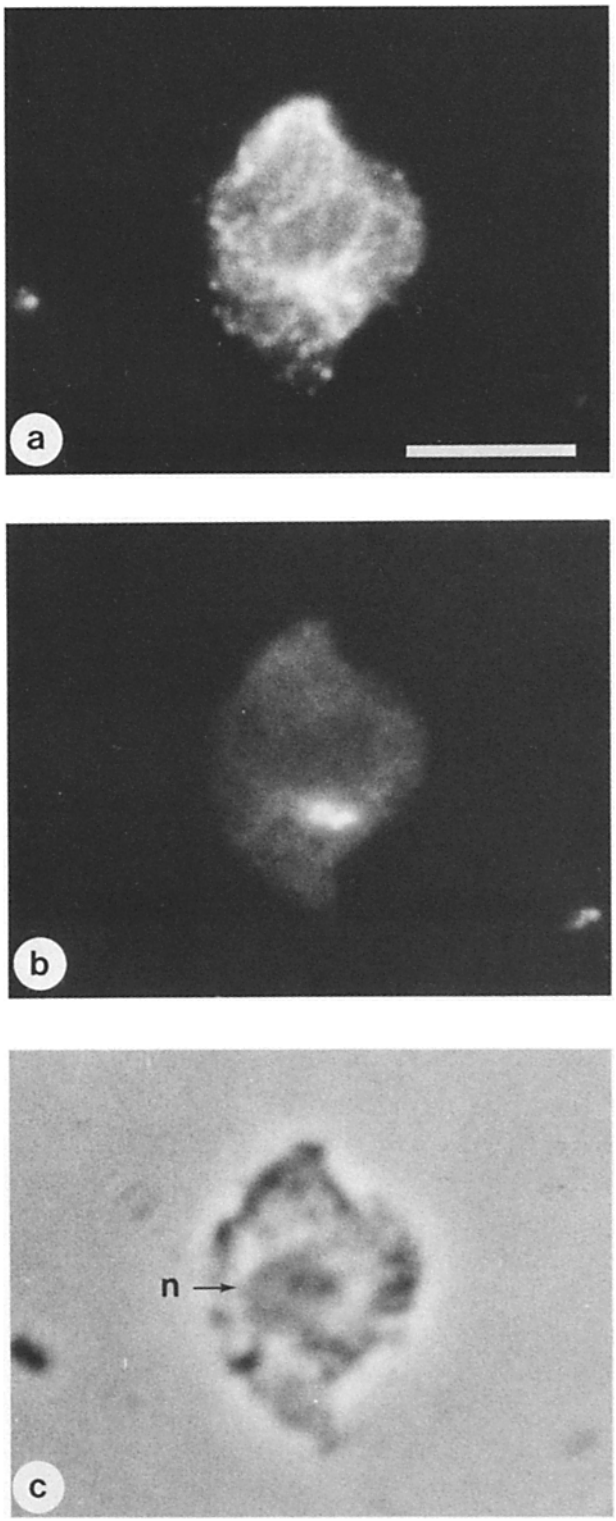


Figure 3. Interphase growing amoeba stained by double-label indirect immunofluorescence with KMP-1 and 6-11B-1. (*a*) Fluorescence photomicrograph of KMP-1-Texas Red staining pattern shows KMP-1 recognizes the MTOC and the cytoskeleton (only part of which is visible in any one focal plane in intact amoebae); (*b*) fluorescence photomicrograph of 6-11B-1-fluorescein staining pattern shows that 6-11B-1 recognizes a small structure in the center of the amoeba that corresponds in location to the MTOC recognized by KMP-1; (*c*) phase contrast photomicrograph shows the location of the nucleus (*n*). Bar, 2 μm.

amoebae. To determine whether acetylated tubulin is specifically localized in certain microtubular structures, immunofluorescence staining patterns of flagellates and of growing amoebae were compared.

Acetylated Alpha-Tubulin Is Localized in the Flagella and Flagellar Cone of Flagellates

Flagellates were fixed in 3.7% formaldehyde and examined by double-label immunofluorescence with two monoclonal antibodies: 6-11B-1, an IgG antibody specific for acetylated alpha-tubulin, and KMP-1, an IgM antibody specific for *Physarum* alpha-tubulin. A typical example of a double-labeled flagellate is presented in Fig. 2. Panel *a* shows the KMP-1 staining pattern, panel *b* shows the 6-11B-1 staining pattern, and panel *c* is the phase contrast photomicrograph. Both KMP-1 and 6-11B-1 recognize proteins in the flagella and the flagellar cone. In both panels *a* and *b* the staining occurs with the long and short flagella as well as the supporting structure. The phase contrast micrograph (*c*) reveals that the flagella and their supporting structure are located close to the nucleus, as observed previously (28).

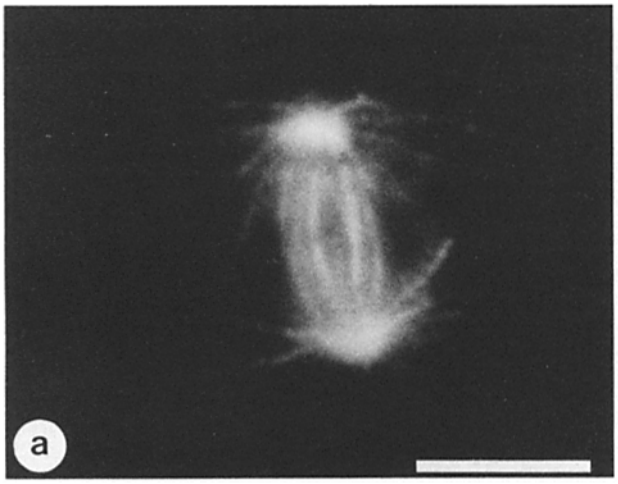
Acetylated Alpha-Tubulin Is Associated with the MTOCs of Growing Amoebae

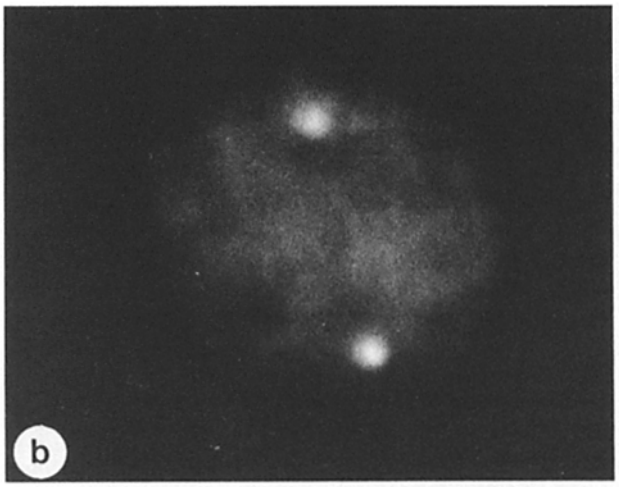
The induction of flagellate formation was avoided by fixing CLd amoebae on the plates on which they were growing, or by using an axenic strain of amoebae grown in axenic medium. Double-label immunofluorescence of amoebae reveals strikingly different staining patterns of 6-11B-1 versus KMP-1, as can be seen in Figs. 3 and 4. KMP-1, the *Physarum*-specific alpha-tubulin antibody, stains the cytoskeleton and the MTOC, which is located near the nucleus (Fig. 3 *a*). By contrast, 6-11B-1 recognizes a small structure, also located near the nucleus (Fig. 3 *b*). Double-labeling of interphase amoebae shows that the two close dots recognized by 6-11B-1 are located within the MTOC recognized by KMP-1.

Mitotic amoebae have only one detectable microtubular structure, the mitotic spindle. Again, amoebae labeled with 6-11B-1 exhibit a staining pattern different from those labeled with KMP-1. KMP-1 labels the whole spindle (Fig. 4 *a*), but 6-11B-1 labels only two widely separated dots (Fig. 4 *b*). Double-label experiments show that the structures reacting with 6-11B-1 are located at the tips of the KMP-1–labeled spindle and correspond to the spindle poles.

No Reaction with 6-11B-1 Is Observed in the Plasmodium by either Western Blots or Immunofluorescence

Microtubular structures are detected in plasmodia only during a short period preceding and during mitosis (11); therefore, our attention was concentrated on this stage of the cell cycle. Synchronous plasmodia were cut in half ~1 h before mitosis as judged by nucleolus position. Half of a plasmodium was labeled with [^{35}S]methionine; the other half was monitored for progress through mitosis both by phase contrast microscopy and by immunofluorescence. The labeled plasmodia were lysed during different points in mitosis and the labeled proteins were electrophoresed on two-dimensional gels for analysis by Western blots. The antibodies used were KMX-1 (a monoclonal antibody that reacts with beta-tubulin) and 6-11B-1. 12 samples were taken at different times





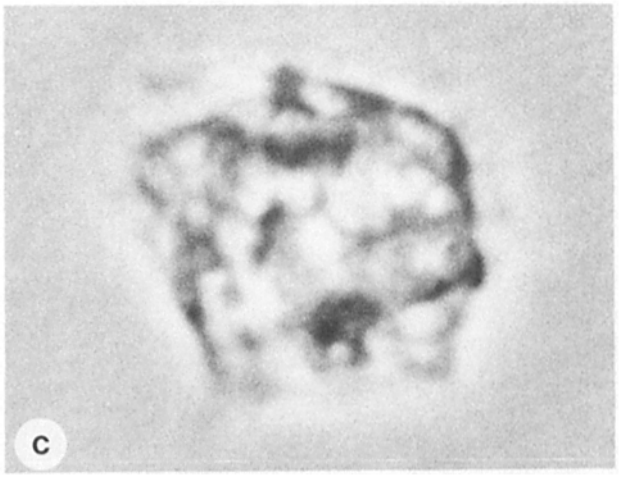
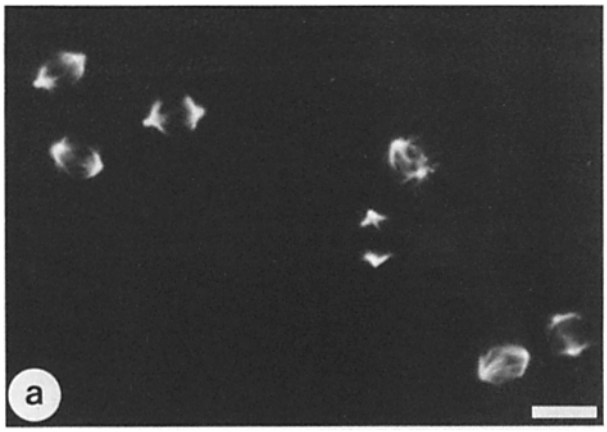


Figure 4. Mitotic amoeba stained by double-label immunofluorescence with KMP-1 and 6-11B-1. (*a*) Fluorescence photomicrograph of KMP-1-Texas Red staining pattern shows that KMP-1 recognizes the mitotic spindle; (*b*) fluorescence photomicrograph of 6-11B-1-fluorescein staining pattern shows that 6-11B-1 recognizes two widely separated dots that correspond in location to the spindle poles; (*c*) phase contrast photomicrograph. Bar, 2 μm.



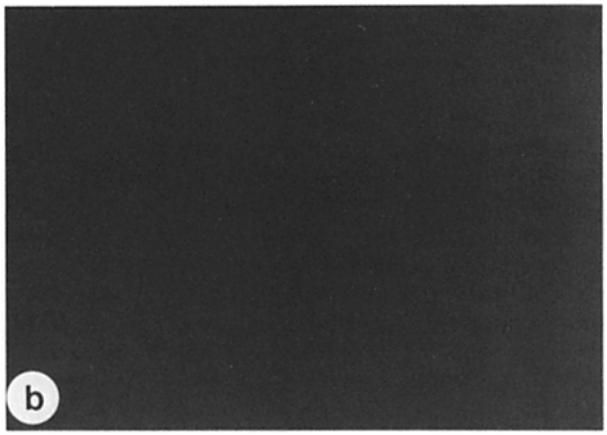


Figure 5. Mitotic plasmodium stained by single-label indirect immunofluorescence with either KMX-1 or 6-11B-1. (*a*) Fluorescence photomicrograph of KMX-1-fluorescein staining pattern shows that KMX-1 recognizes the mitotic spindle. Exposure, 60 s. (*b*) Fluorescence photomicrograph of 6-11B-1-fluorescein staining pattern shows that 6-11B-1 does not react with the mitotic spindle. Exposure, 60 s. The photomicrographs in *a* and *b* were developed and printed identically.

during plasmodial mitosis. At each time point, a signal was observed with KMX-1, but not with 6-11B-1. Examination of autoradiographs of the radioactive blots demonstrated that all the plasmodial tubulins were present (data not shown).

Plasmodial immunofluorescence slides were made concurrently with samples for two-dimensional gel analysis. These samples served two purposes: to determine the mitotic stage of the plasmodium comprising the gel sample and to assay the presence or absence of the 6-11B-1 antigen by an independent method. No signal was detected when any stage of mitosis was assayed with 6-11B-1, although the positive control (KMX-1) reacted with the spindle during all stages of mitosis (Fig. 5).

Discussion

The distribution of acetylated alpha-tubulin throughout three developmental stages in *Physarum polycephalum* has been studied. Acetylated alpha-tubulin is present in the flagellate and in the amoeba, but is not detected in the plasmodium. There are several possible explanations for the lack of reaction of the plasmodial samples with the 6-11B-1 antibody.

First, the level of acetylated alpha-tubulin may lie below the detection limits of the assays employed in this study. Second, the acetylation of alpha-tubulin may be transient; a short expression time would minimize the chance of detection. Third, the plasmodium may lack the enzymes or tubulins necessary for acetylation of alpha-tubulin.

It has been previously shown that a posttranslational modification occurs during *Physarum* flagellate formation (10). The evidence presented here suggests that this modification includes acetylation of alpha-tubulin. The acetylation of alpha-tubulin during flagellate formation has also been observed in *Chlamydomonas* (16).

Acetylated alpha-tubulin is present in all of the flagellate's microtubular structures but only in certain of the growing amoeba's microtubular structures. In the flagellate, the flagella and their supporting structure are recognized by both KMP-1 and 6-11B-1. In the growing amoeba the MTOC also reacts with both KMP-1 and 6-11B-1. The amoebal cytoskeleton and most of the mitotic spindle are recognized only by KMP-1. At the level of the light microscope it is impossible to identify precisely which parts of these microtubular structures are being labeled by each of these antibodies. For example, is acetylated alpha-tubulin located only in the centrioles or in the pericentriolar material of the amoebal MTOC? This uncertainty might be resolved by electron microscope immunocytochemistry.

These experiments have yielded two unexpected results. The first is that KMP-1 seems to react more strongly with α1- than with α3-tubulin on Western blots. The autoradiograph in Fig. 1 was in the linear range of the film. Assuming that both α1 and α3 were equally labeled over the 3-d labeling period, the protein pattern observed on the autoradiograph indicates that there are equal amounts of α1 and α3. By contrast, the immunoblot with KMP-1 indicates that this antibody reacts more strongly with α1 than with α3. One explanation would be that the KMP-1 epitope is altered during acetylation. A second explanation would be that two gene products comprise the α1-isotype spot and that only one of these can be acetylated. KMP-1 would react strongly with the gene product that could not be acetylated and weakly, if at all, with the hypothesized second gene product. The fact that KMP-1 binds selectively to α1-tubulin in *Physarum* flagellates and not to α3 or to chemically acetylated myxamoebal tubulin, has been communicated to us by C. R. Birkett and K. Gull, University of Kent at Canterbury, UK (20*a*).

A second unexpected and potentially important result is that, in the growing amoeba, acetylated alpha-tubulin is associated with a particular microtubular structure. Several lines of evidence indicate that this structure is within the MTOC. First, it is located near the nucleus and corresponds in location to the MTOC at the center of the KMP-1-labeled cytoskeleton. Second, these structures are found to move apart immediately before mitosis. In double-label experiments, the KMP-1 staining shows that the cytoskeleton has broken down and the MTOC has split. The 6-11B-1 staining demonstrates two distinct dots, corresponding in location to the MTOC. At this stage in the amoebal cell cycle, the nuclear membrane is still intact. This finding is in keeping with the observations that the MTOC divides before mitosis in other systems (13). The exclusive localization of a well-defined antigen within the MTOC of growing amoebae may be important in that it could offer a unique opportunity to study this structure.

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