

Microtubules Containing Acetylated α -Tubulin in Mammalian Cells in Culture

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Abstract. The subcellular distribution of microtubules containing acetylated α -tubulin in mammalian cells in culture was analyzed with 6-11B-1, a monoclonal antibody specific for acetylated α -tubulin. Cultures of 3T3, HeLa, and PtK₂ cells were grown on coverslips and observed by immunofluorescence microscopy after double-staining by 6-11B-1 and B-5-1-2, a monoclonal antibody specific for all α -tubulins. The antibody 6-11B-1 binds to primary cilia, centrioles, mitotic spindles, midbodies, and to subsets of cytoplasmic microtubules in 3T3 and HeLa cells, but not in PtK₂ cells. These observations confirm that the acetylation of α -tubulin is a modification occurring in different microtubule structures and in a variety of eukaryotic cells. Some features of the acetylation of cytoplasmic microtubules of mammalian cells are also described here. First, acetylated α -tubulin is present in microtubules that, under depolymerizing conditions, are more

stable than the majority of cytoplasmic microtubules. In addition to the specific microtubule frameworks already mentioned, cytoplasmic microtubules resistant to nocodazole or colchicine, but not cold-resistant microtubules, contain more acetylated α -tubulin than the rest of cellular microtubules. Second, the α -tubulin in all cytoplasmic microtubules of 3T3 and HeLa cells becomes acetylated in the presence of taxol, a drug that stabilizes microtubules. Third, acetylation and deacetylation of cytoplasmic microtubules are reversible in cells released from exposure to 0°C or antimetabolic drugs. Fourth, the epitope recognized by the antibody 6-11B-1 is not absolutely necessary for cell growth and division. This epitope is absent in PtK₂ cells. The acetylation of α -tubulin could regulate the presence of microtubules in specific intracellular spaces by selective stabilization.

THE majority of α -tubulin assembled in the axonemes of *Chlamydomonas* flagella is acetylated on the ϵ -amino group of a lysine residue (6). Acetylation of this lysine is catalyzed in the flagellar matrix (5, 3), and deacetylation occurs in the cell body when flagella are resorbed (7). On the basis of these observations, LHernault and Rosenbaum proposed that acetylation and deacetylation of α -tubulin have a function in regulating the assembly of axonemal microtubules. Subsequent studies, including this one, showed that acetylated α -tubulin is present in various microtubule structures and may have a role in stabilizing the structure of all microtubules.

The analysis of interphase *Chlamydomonas* cells by a monoclonal antibody specific for acetylated α -tubulin provided evidence for the presence of the antigen in basal bodies and in subsets of cytoplasmic microtubules (8). The same antigen was detected in cilia and flagella of a variety of cells (12). Cytoplasmic microtubules containing acetylated α -tubulin in *Chlamydomonas* were found to be more resistant than other cellular microtubules to the effects of antimetabolic drugs (8). As acetylated α -tubulin was found in three microtubule structures of *Chlamydomonas* and in axonemes of various cells, we wanted to investigate whether the modified subunit is also present in cytoplasmic microtubules

of other cell types and in different microtubule frameworks. A more general model of the function of the acetylation could result from this study. We report here the analysis of three cell lines, 3T3, HeLa, and PtK₂, by two monoclonal antibodies, one specific for acetylated α -tubulin, the other specific for all α -tubulins.

Materials and Methods

Cell Culture

3T3, fibroblast-like cells from mouse embryo, were provided by Dr. David Foster (Rockefeller University, NY); HeLa, epithelial-like cells from epitheloid carcinoma of human cervix, were provided by Dr. Nathaniel Heintz (Rockefeller University); and PtK₂, epithelial-like cells from kidney of *Potorous tridactylis*, were obtained from the American Type Culture Collection (cat. No. CCL 56; Rockville, MD). Cells were grown on glass coverslips in MEM supplemented with 10% FCS, penicillin, and streptomycin (100 U/ml) at 37°C in 5% CO₂. Subcultures of cells were performed at a density of 1.6×10^4 or 0.3×10^4 cells/35-mm dish 2 or 3 d before staining. Cells used for experiments of antibody binding to soluble proteins were collected from confluent cultures. In some experiments culture medium was substituted with fresh medium containing 10 μ M taxol (Natural Products Branch of National Cancer Institute, Bethesda, MD) or 10 μ g/ml cycloheximide (Sigma Chemical Co., St. Louis, MO), or 0.1 or 1 μ g/ml nocodazole (Aldrich Chemical Co., Gillingham, England), or 10^{-6} M colchicine (Al-

drich Chemical Co.). Recovery from exposure to nocodazole, colchicine, or taxol was performed by washing the coverslips once in medium and placing them in new 35-mm culture dishes containing 4 ml of fresh medium at 37°C. Recovery from exposure to 0°C was performed without substitution of medium. The 35-mm culture dishes, containing 4 ml of medium, and the coverslips were warmed to 37°C in 15 min.

Monoclonal Antibodies

The monoclonal antibodies 6-11B-1, B-5-1-2, and C-241-2 were used in culture medium or as pure IgGs. IgGs were purified from serum-free culture medium (HB 102 medium, New England Nuclear, Boston, MA) by Protein A-Sepharose affinity chromatography. The antibody 6-11B-1 is specific for acetylated α -tubulin (12) and antibody C-241-2 is specific for the β -subunit of dynein from the outer arms of *Strongylocentrotus purpuratus* sperm axonemes (11). Antibody B-5-1-2 recognizes all α -tubulin isoforms in *Chlamydomonas*, sea urchin, and human tubulin. The hybridoma B-5-1-2 produces an IgG and was selected among hybridomas derived from the fusion of the myeloma P₃U₁ with mouse spleen cells, as previously described. The mouse was immunized with Sarkosyl-resistant filaments (9) from *S. purpuratus* sperm axonemes. Four hybridomas secreting monoclonal antibodies against α -tubulin were isolated. The antibody B-5-1-2 was selected because it has high affinity and reacts with all α -tubulin isoforms (Chang, X.-J., and G. Piperno, unpublished results).

Immunofluorescence

Cells on coverslips were exposed to 1% Triton X-100, 2 mM EGTA, 5 mM Pipes (Sigma Chemical Co.), pH 6.7, for 1 min at room temperature, then placed in methanol cooled on solid CO₂ for 5 min, warmed for 5 min, and rehydrated for 5 min in 0.13 M NaCl, 0.01 M sodium phosphate, pH 6.8 (PBS). Chemical acetylation in situ was performed by exposing methanol fixed cells to 0.1% acetic anhydride, 1 M sodium phosphate, pH 8, for 1 min before rehydrating and washing them in PBS. The incubation of cells with antibody 6-11B-1 was performed overnight at 8°C. Biotin-labeled sheep antimouse antiserum (Amersham Corp., Arlington Heights, IL; diluted 1:25), antibody B-5-1-2, and fluorescein-labeled goat antimouse (Cappel Laboratory, Malvern, PA; diluted 1:50), Texas Red Streptavidin (Amersham Corp.; diluted 1:25), were subsequently applied to the cells for 1 to 2 h at room temperature. All dilutions were in 0.1% BSA in PBS. Incubations were performed in a humid chamber. Three washings in PBS for 7 min were performed with shaking after each incubation. Coverslips were then inverted on a slide over a drop of 90% vol/vol glycerol, 10% vol/vol PBS, 1 mg/ml *p*-phenylene diamine (pH 7.15), and maintained in place with nail polish.

Controls of the permeabilization and fixation procedures were performed on 3T3 cells. Prolonging the time of exposure to the Triton X-100 solution or inverting the order of the operations concerning the exposure to Triton and to methanol or fixing the cells with 1% glutaraldehyde in 100 mM Tris/HCl (pH 7.5), 15 mM EGTA, and 2.5 mM MgCl₂ did not alter the staining patterns of antibodies 6-11B-1 or B-5-1-2. However each of these procedures produced labeling of microtubules that was less contrasted over the background than that shown in the Results section.

Controls of specificity of the antibody binding included the application of antibody C-241-2 as negative control, single-labeling procedures with antibody 6-11B-1 or B-5-1-2, and application of pure 6-11B-1 or C-241-2 IgGs up to concentrations of 50 μ g/ml. In each case it was confirmed that the staining patterns obtained with 6-11B-1 or B-5-1-2 were specific and were not altered by the double-staining procedure or by the presence of culture medium.

Photomicrographs were obtained using a Zeiss Photomicroscope II equipped with an epi-fluorescence condenser III RS and a 63 X Planapo objective. The exposure times varied from 30 to 90 s. The film, Kodak Tri X Pan, was developed in D76 developer.

Electrophoresis, Immunoblotting, and Dot Immunobinding

Protein samples from 3T3 cells grown overnight in the presence or absence of 10 μ M taxol were prepared by scraping the cells from the dishes in PBS containing 1 mM EDTA. After sedimentation the cells were suspended in 0.1 ml per plate of a solution containing 0.1 mM phenyl methyl-sulfonyl fluoride (PMSF) and 0.1 mg/ml soybean trypsin inhibitor. The suspension was sonicated 15 s at 50 W, then SDS was added to the concentration of 1%. The samples were boiled and immediately used for electrophoresis or for dot binding assays.

To prepare fractions containing unpolymerized or polymerized tubulin from taxol-treated 3T3 cells, cells were grown, collected, and washed as described above. The cell pellet was then resuspended in 10 vol of 0.5% NP-40, 0.14 M NaCl, 2 mM MgSO₄, 2 mM EGTA, 10 mM Tris/Cl pH 7.6, 10 μ M taxol, 0.1 mM PMSF, 0.1 mg/ml soybean trypsin inhibitor, and left on ice for 10 min. The suspension was then centrifuged for 30 min at 37°C and 20,600 rpm in a rotor (model SW65; Beckman Instruments, Inc., Fullerton, CA). The pellet was resuspended by sonication as described. Both supernatants and pellets were prepared for analysis as described. Control experiments, performed on untreated 3T3 cells, indicated that under these conditions the addition of taxol during and after the lysis did not induce the acetylation of α -tubulin or the polymerization of microtubules.

Protein samples from PtK₂ cells were prepared from cultures treated with 10 μ g/ml nocodazole for 30 min. Cells were detached by adding 3 ml per 100 mm plate of a medium containing 0.25% trypsin and 10 μ g/ml nocodazole for 8 min, at 37°C. The trypsin was removed and the cells were collected in medium containing 10 μ g/ml nocodazole. After sedimentation and a wash in PBS containing 10 μ g/ml nocodazole and 0.1 mg/ml soybean trypsin inhibitor, the cell pellet was suspended in 5 vol of 0.14 M NaCl, 1.5 mM MgCl₂, 10 mM Tris/Cl, pH 8.6. Then NP-40 was added to a final concentration of 0.5% and the suspension was left on ice for 10 min before being spun down at 12,000 g for 5 min. The pellet was discarded and the supernatant was diluted 1:1 with 2 M sodium phosphate, pH 8.0. One-half of the sample was acetylated in vitro by adding 1% vol/vol acetic anhydride for 10 min at room temperature. SDS was added to a final concentration of 0.1% to acetylated and unacetylated samples, which were then dialyzed against 0.1% SDS, 15 mM β -mercapto-ethanol, 1 mM EDTA, 40 mM Tris/Borate, pH 8.65, and analyzed as described above.

Electrophoresis of polypeptides, transfer to nitrocellulose, and detection of antigens by monoclonal antibodies was performed as described previously (11). Samples were processed for quantitative dot-immunobinding assay as described by Jahn et al. (4) and modified by Piperno and Fuller (12).

Results

Subsets of Cytoplasmic Microtubules and Specific Microtubule Frameworks Contain Acetylated α -Tubulin

We observed by fluorescence microscopy 3T3, HeLa, and PtK₂ cells that were grown on glass coverslips and processed for double-label immunofluorescence, as described in Materials and Methods. Double-label staining of microtubules was performed with 6-11B-1, a monoclonal antibody specific for acetylated α -tubulin, and with B-5-1-2, a monoclonal antibody that recognizes all α -tubulin isoforms in a variety of organisms.

Subcellular locations of all microtubules in interphase 3T3, HeLa, and PtK₂ cells are shown in Fig. 1, *a'*, *b'*, and *c'*. Although the length, shape, and density of microtubules give each cell line a characteristic appearance, classical patterns of microtubules radiating from the perinuclear region toward the edge of the cells are visible in 3T3 cells (Fig. 1 *a'*), HeLa cells (Fig. 1 *b'*), and PtK₂ cells (Fig. 1 *c'*). Microtubules containing acetylated α -tubulin are present in 3T3 cells (Fig. 1 *a*), and HeLa cells (Fig. 1 *b*), but are not seen in PtK₂ cells (Fig. 1 *c*). In 3T3 cells, acetylated α -tubulin is found in segments of a few microtubules (see cross in Fig. 1 *a*) and in short structures radiating from the centrosphere (see arrows in Fig. 1 *a*). These structures appear to be primary cilia. In some areas of the coverslips where 3T3 cells were confluent, a higher amount of microtubules containing acetylated α -tubulin was observed. In HeLa cells, acetylated α -tubulin is assembled in microtubules that seem to be concentrated about the cell center: some are curved and not oriented toward the centrosphere (Fig. 1 *b*).

Cells undergoing mitosis or cytokinesis were also examined. Again 3T3 and HeLa, but not PtK₂ cells, were labeled

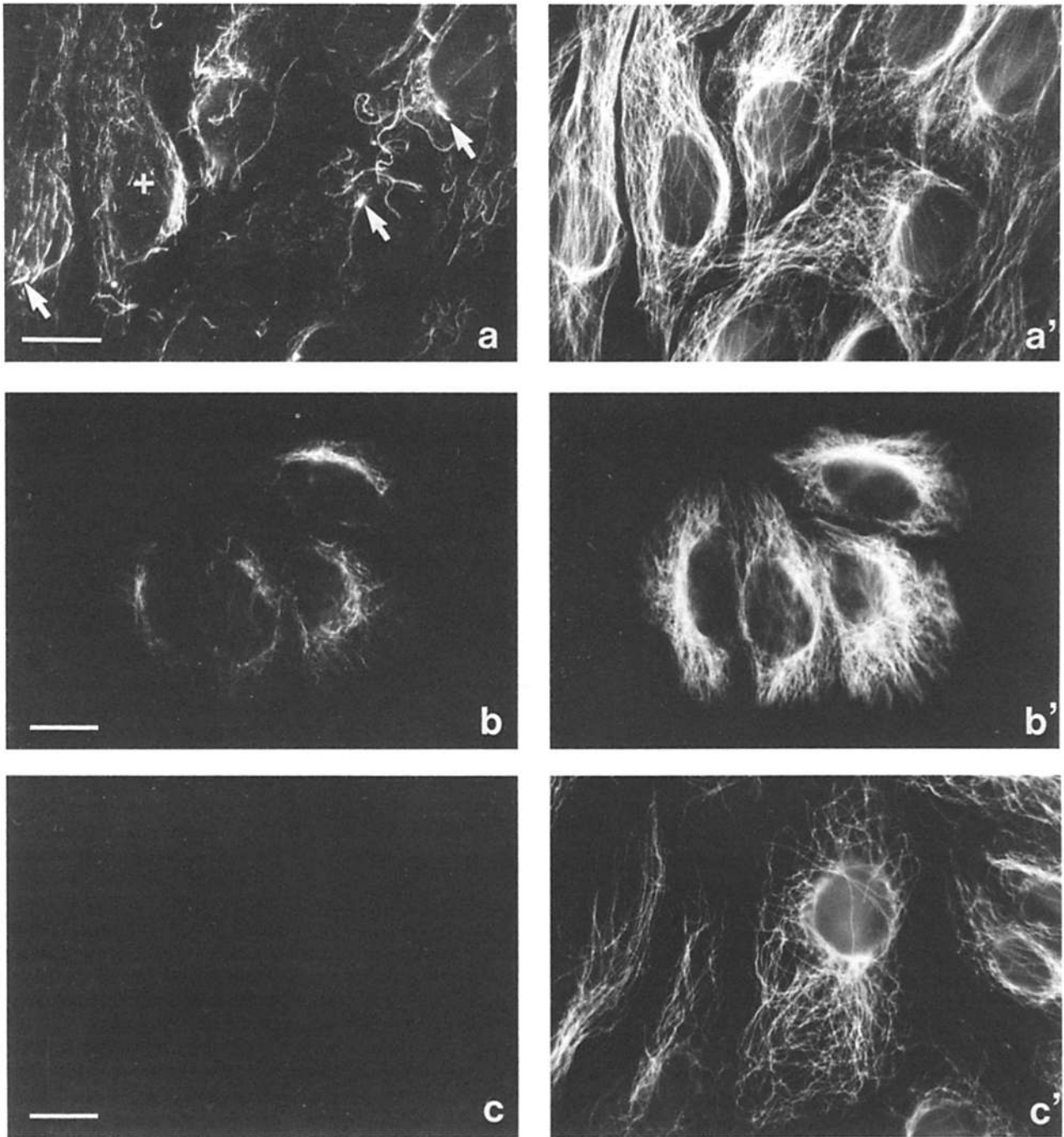


Figure 1. Anti-tubulin double-label immunofluorescence of cultured interphase cells. (a) 3T3 cells stained by 6-11B-1, a monoclonal antibody specific for acetylated α -tubulin. Arrows indicate primary cilia and a cross indicates microtubules forming a fork and binding the antibody only on segments of their structure. (a') 3T3 cells stained by B-5-1-2, a monoclonal antibody specific for all α -tubulin isoforms. (b) HeLa cells stained by 6-11B-1. (b') HeLa cells stained by B-5-1-2. (c) No staining was obtained by 6-11B-1 on PtK₂ cells. (c') PtK₂ cells stained by B-5-1-2. Bars, 10 μ m.

by the antibody 6-11B-1, which is specific for acetylated α -tubulin. Mitotic spindles of 3T3 cells (Fig. 2, *a* and *a'*) and HeLa cells (Fig. 2, *b* and *b'*) were stained by both antibodies 6-11B-1 and B-5-1-2. Therefore, polar regions, kinetochores, and continuous fibers of the spindles contain acetylated α -tubulin. The same antigen is present in midbodies of 3T3 cells (Fig. 2 *c*) and HeLa cells (Fig. 2 *d*).

In summary, subsets of cytoplasmic microtubules and well defined microtubule structures such as primary cilia, mitotic spindles, and midbodies contain acetylated α -tubulin in 3T3 and HeLa cells. In PtK₂ cells the epitope recognized by the monoclonal antibody, 6-11B-1, is either absent or masked. The experiments performed to test these hypotheses are described in the last section of Results.

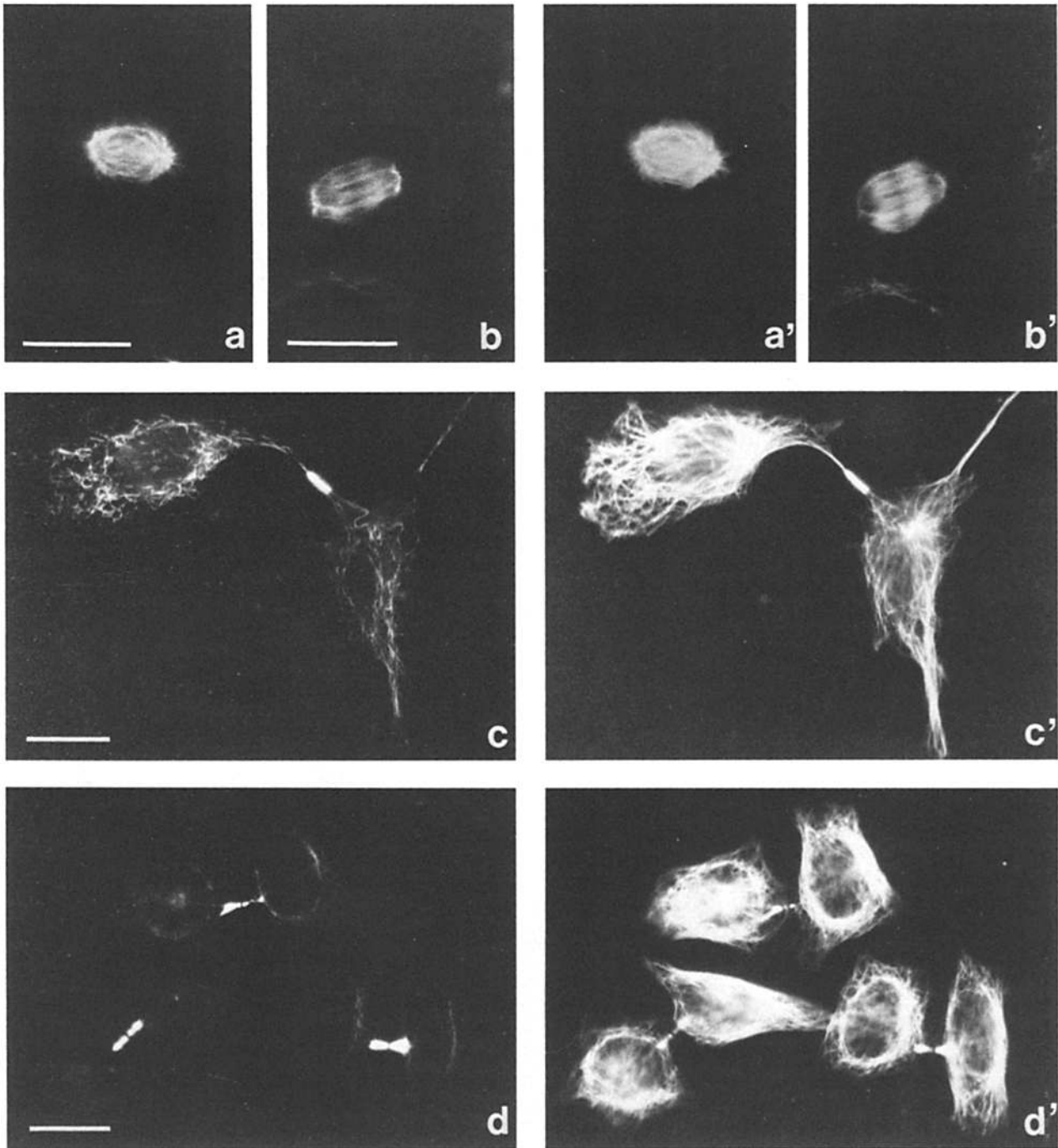


Figure 2. Anti-tubulin double-label immunofluorescence of 3T3 and HeLa cells at mitosis and cytokinesis. (a) Mitotic spindle of a 3T3 cell stained by 6-11B-1, a monoclonal antibody specific for acetylated α -tubulin. (b) Mitotic spindle of a HeLa cell stained by 6-11B-1. (a') Same as in a, stained by B-5-1-2, a monoclonal antibody specific for all α -tubulin isoforms. (b') Same as in b, stained by B-5-1-2. (c) 3T3 cells at cytokinesis stained by 6-11B-1. (c') Same as in c, stained by B-5-1-2. (d) HeLa cells at cytokinesis, stained by 6-11B-1. (d') Same as in d, stained by B-5-1-2. Bars, 10 μ m.

Taxol-stabilized Microtubules Become Acetylated on α -Tubulin

Microtubules found in the structure of primary cilia and midbodies differ in two properties from the majority of cytoplasmic microtubules. First, they form regular frameworks that contain various appendages. Second, they are more stable than other cytoplasmic microtubules under conditions that promote microtubule disassembly (2). Cellular structures

that cross-link the microtubules along their length or cap their ends may cause this stabilization (2). Formation of ordered structures and stability of microtubules have also been correlated with the presence of acetylated α -tubulin in the microtubules of *Chlamydomonas* (8). We wanted to investigate whether this correlation is verified for cytoplasmic microtubules that are stabilized by taxol, an antimetabolic drug that induces microtubule assembly and formation of micro-

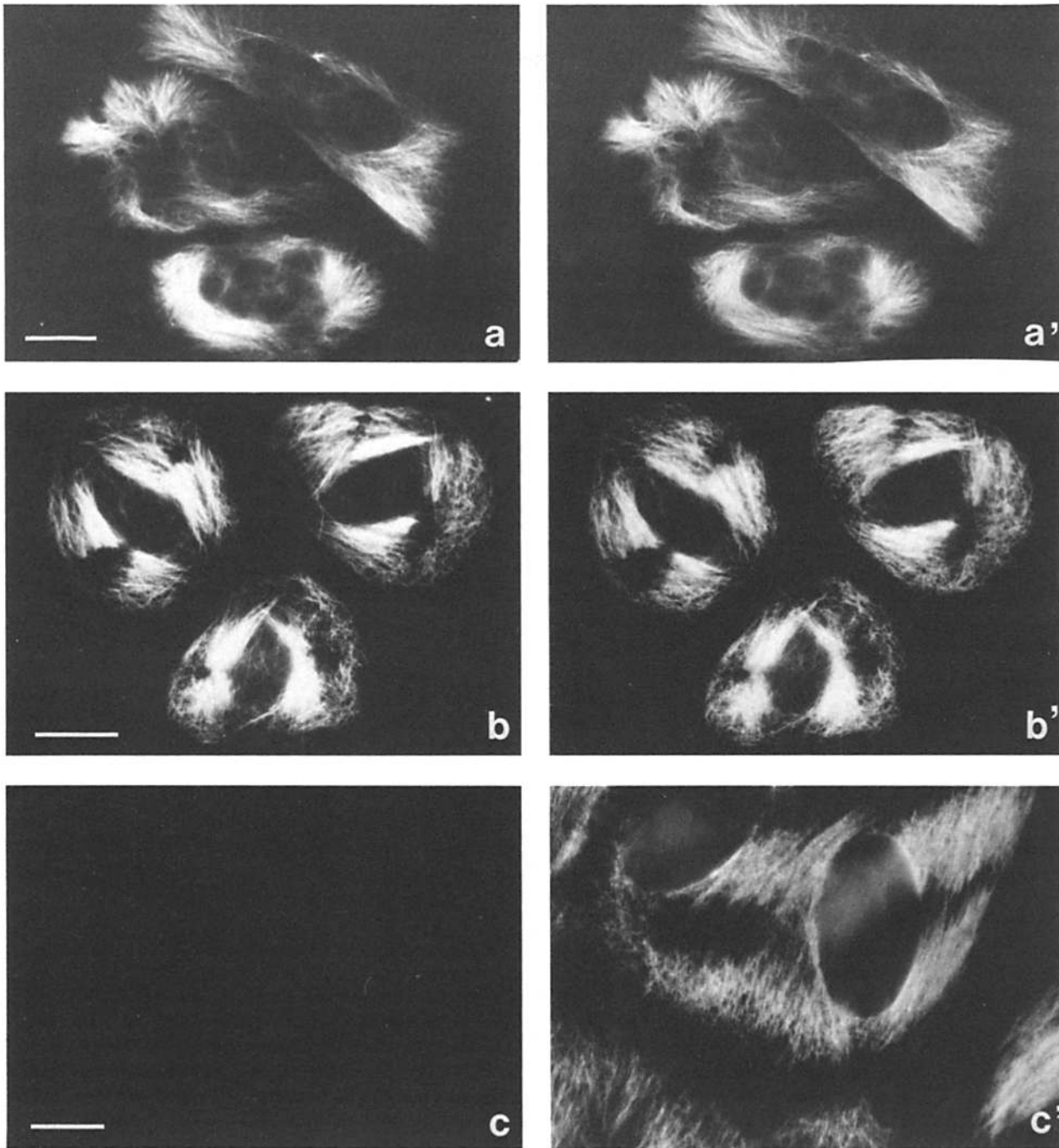


Figure 3. Anti-tubulin double-label immunofluorescence of cells cultured overnight in the presence of 10 μM taxol. (a) 3T3 cells stained by 6-11B-1, a monoclonal antibody specific for acetylated α -tubulin. (a') Same as in a, stained by B-5-1-2, a monoclonal antibody specific for all α -tubulin isoforms. (b) HeLa cells stained by 6-11B-1. (b') Same as in b, stained by B-5-1-2. (c) No staining was obtained by 6-11B-1 on PtK₂ cells. (c') PtK₂ cells stained by B-5-1-2. Bars, 10 μm .

tubule bundles (10, 13). Cytoplasmic microtubules, which normally disassemble and assemble at high rates, in the presence of taxol bind the drug and are stabilized (13).

Cells that were cultured overnight in the presence of 10 μM taxol were processed for double-label immunofluorescence microscopy, as described before. 3T3 cells are shown in Fig. 3, a and a', HeLa cells in Fig. 3, b and b', and PtK₂ cells in Fig. 3, c and c'. The staining of all microtubules by B-5-1-2 (Fig. 3, a', b', and c') reveals that bundles of microtubules were formed in each cell line. The staining with the an-

tibody 6-11B-1, against acetylated α -tubulin, is nearly identical to that obtained by B-5-1-2 in 3T3 and HeLa cells, but is completely absent in PtK₂ cells (Fig. 3, a-c). These results show that an increase of polymerized, acetylated α -tubulin is induced in the presence of taxol in 3T3 and HeLa cells. Taxol bound to microtubules of PtK₂ cells because it formed bundles of microtubules. However, even under this condition, the antibody 6-11B-1 does not recognize any epitope in PtK₂ cells.

The effect of exposure to taxol was also analyzed by bind-

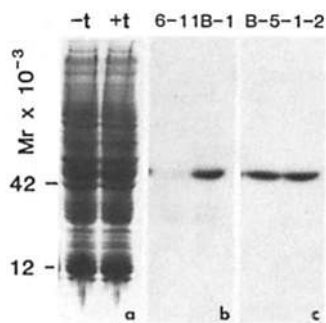


Figure 4. Antibody binding to α -tubulins of 3T3 cells cultured overnight in absence or in presence of 10 μ M taxol. (a) Coomassie Blue-stained polypeptides from whole cells after separation by SDS polyacrylamide gel electrophoresis. The position of molecular weight standards is indicated at the left. Left lane marked $-t$, contains polypeptides from cells cultured in absence of

taxol. Right lane, marked $+t$, contains polypeptides from cells cultured in presence of taxol. Two sets of the same samples were electrophoresed in parallel and then transferred to nitrocellulose. (b) Autoradiogram of the nitrocellulose incubated with 6-11B-1, a monoclonal antibody specific for acetylated α -tubulin. (c) Autoradiogram of the nitrocellulose incubated with B-5-1-2, a monoclonal antibody specific for all α -tubulin isoforms.

ing experiments of either of two antibodies, 6-11B-1 or B-5-1-2, and with preparations of protein from 3T3 cells.

Fig. 4 *a* shows electrophoretograms of $\sim 90 \mu$ g of total cellular protein from confluent 3T3 cells, cultured overnight in the absence or presence of 10 μ M taxol. The patterns are indistinguishable. A large number of unresolved bands are present in both electrophoretograms. Fig. 4, *b* and *c* shows immunoblots of the same samples incubated with 6-11B-1 or B-5-1-2. The antibody 6-11B-1 identifies one major band of acetylated α -tubulin in the lane containing proteins from taxol-treated cells and a faint band in the other lane. In contrast, the antibody B-5-1-2 identifies one band of α -tubulin that is equally intense in each lane. Therefore the two samples of protein contain approximately equal amounts of α -tubulin. This α -tubulin is acetylated at a low level in untreated cells and becomes more acetylated upon exposure of cells to taxol.

The same conclusions were reached by dot assay on increasing amounts of proteins from treated and untreated cells. Table I shows that the binding of the antibody 6-11B-1 to proteins of 3T3 cells increased severalfold after treatment of the cells with taxol. Since the immunoblot shown in Fig. 4 *b* confirmed the monospecificity of the antibody 6-11B-1, this increased binding indicates that the concentration of acetylated α -tubulin rises in these cells. Taxol treatment also raised the total concentration of α -tubulin in the cells, as evidenced by the increased binding of the antibody B-5-1-2. However, the relative increase of total α -tubulin is much lower than that of acetylated α -tubulin. These results indicate that upon exposure to taxol, acetylation of α -tubulin occurs. An increase in the acetylation of α -tubulin in taxol-treated 3T3 cells could be detected after 30 min of exposure. Maximal acetylation was reached in 12 h (not shown).

Table I. Binding of Monoclonal Antibodies to α -Tubulins of 3T3 Cells

Treatment	6-11B-1	B-5-1-2
None	4.5 (0.85)	48.6 (0.99)
Taxol (12 h)	12.5 (0.97)	70.5 (0.99)
Taxol (12 h) + recovery (60 min)	4.2 (0.95)	56.1 (0.96)

The radioactivity bound to varying quantities of total protein was measured on three samples for each amount of protein. A linear regression was then performed using a Hewlett-Packard 11C calculator. The numbers are expressed in counts per minute per microgram; they represent the slopes of the regression lines. The numbers in parentheses are the corresponding coefficients of determination r^2 .

Drug-resistant but Not Cold-resistant Microtubules Contain More Acetylated α -Tubulin than the Majority of Cytoplasmic Microtubules

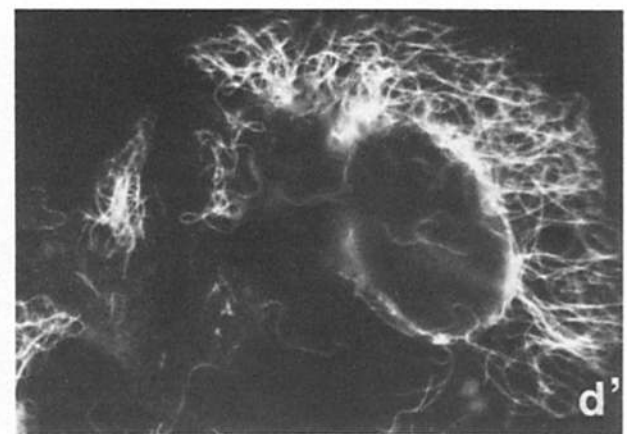
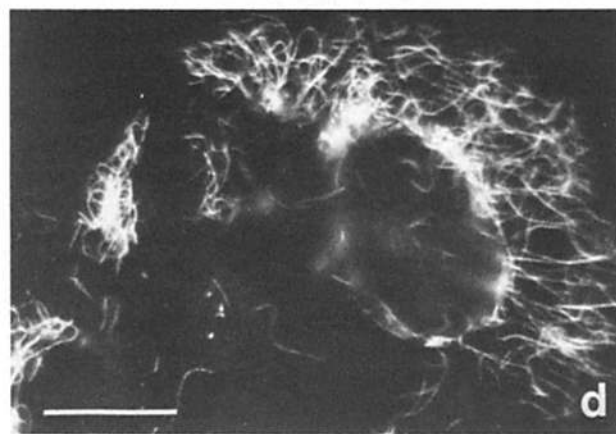
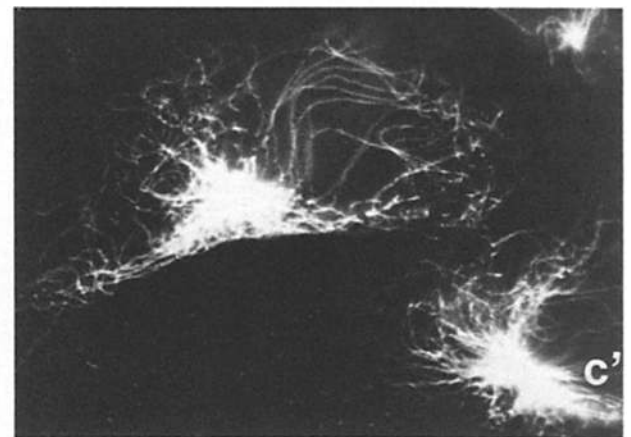
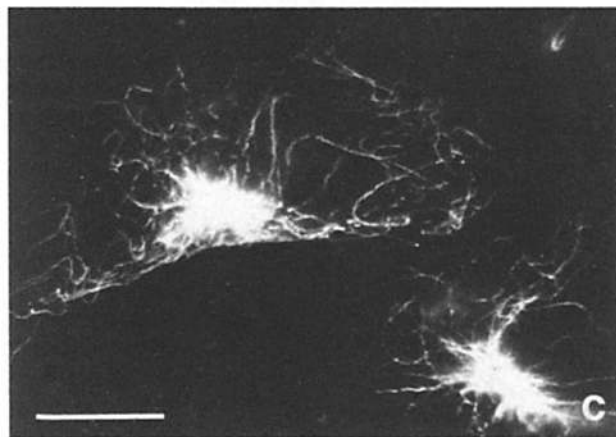
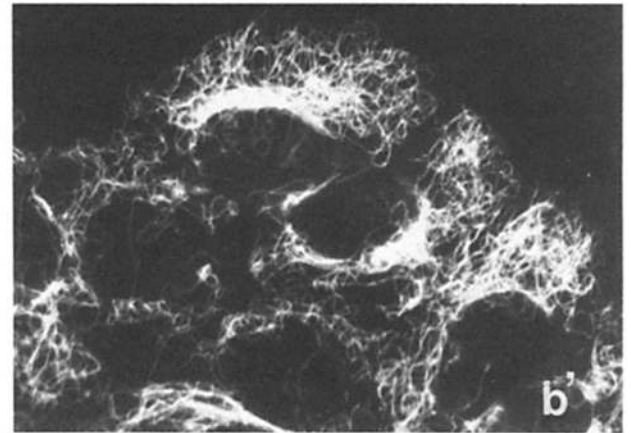
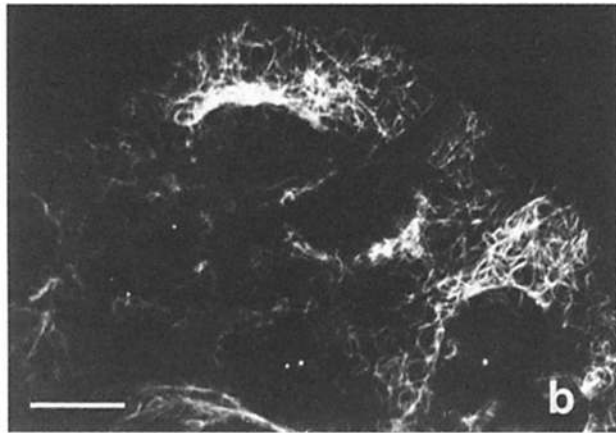
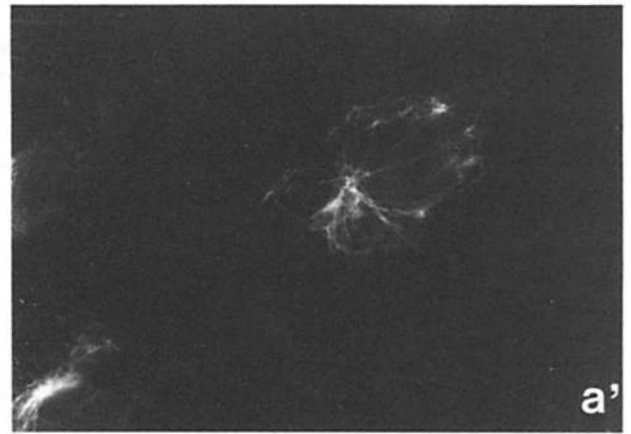
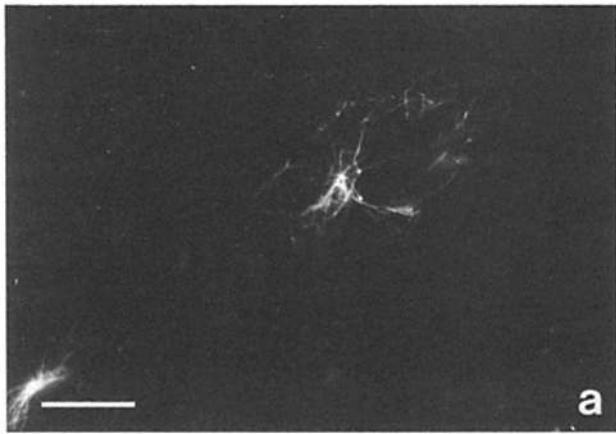
A second approach to the investigation of whether stable cytoplasmic microtubules contain acetylated α -tubulin employed antimetabolic drugs that promote microtubule disassembly. Previous experiments showed that in *Chlamydomonas* cell bodies a subset of cytoplasmic microtubules are acetylated and are much more resistant to drug-induced depolymerization than nonacetylated microtubules (8). We describe here observations performed by immunofluorescence microscopy of 3T3 and HeLa cells after they are exposed to 0.1 μ g/ml nocodazole for 30 min or 10^{-6} M colchicine for 1 h. In all instances the majority of cytoplasmic microtubules were depolymerized and most of the residual microtubules were stained by 6-11B-1, the antibody against acetylated α -tubulin.

Double-label immunofluorescence was performed as previously described. Staining by the antibody 6-11B-1 is shown in Fig. 5, *a-d*. 3T3 cells exposed to nocodazole are shown in Fig. 5, *a* and *a'*. The same cells exposed to colchicine are shown in Fig. 5, *c* and *c'*. In both cases the few microtubules that are distinguishable as single filaments appear to contain acetylated α -tubulin at least in part of their length. Similar patterns of staining are seen in HeLa cells treated with nocodazole (Fig. 5, *b* and *b'*) or colchicine (Fig. 5, *d* and *d'*).

The resistance of these residual microtubules is not absolute. Fig. 6, *a* and *a'* shows that 3T3 cells exposed to 1 μ g/ml nocodazole for 30 min contain few cytoplasmic microtubules and primary cilia. Acetylated α -tubulin is present in these residual structures.

We observed that in *Chlamydomonas* all cytoplasmic microtubules are depolymerized upon exposure to 0°C , independently of their content of acetylated α -tubulin (8). Although exposure of cells to 0°C affects microtubules as well as most cellular structures, and therefore may not be considered a specific treatment for testing microtubule stability, we wanted to investigate whether cold resistant microtubules, which exist in 3T3 and HeLa cells, are enriched in acetylated α -tubulin like the drug-resistant microtubules.

Figure 5. Anti-tubulin double-label immunofluorescence of 3T3 and HeLa cells cultured in presence of nocodazole or colchicine. (a) 3T3 cells exposed to 0.1 μ g/ml nocodazole for 30 min and stained by 6-11B-1, a monoclonal antibody specific for acetylated α -tubulin. (a') Same as in *a*, stained by B-5-1-2, a monoclonal antibody specific for all α -tubulin isoforms. (b) HeLa cells exposed to 0.1 μ g/ml nocodazole for 30 min and stained by 6-11B-1. (b') Same as in *b*, stained by B-5-1-2. (c) 3T3 cells exposed to 10^{-6} M colchicine for 1 h and stained by 6-11B-1. (c') Same as in *c*, stained by B-5-1-2. (d) HeLa cells exposed to 10^{-6} M colchicine for 1 h and stained by 6-11B-1. (d') Same as in *d*, stained by B-5-1-2. Bars, 10 μ m.



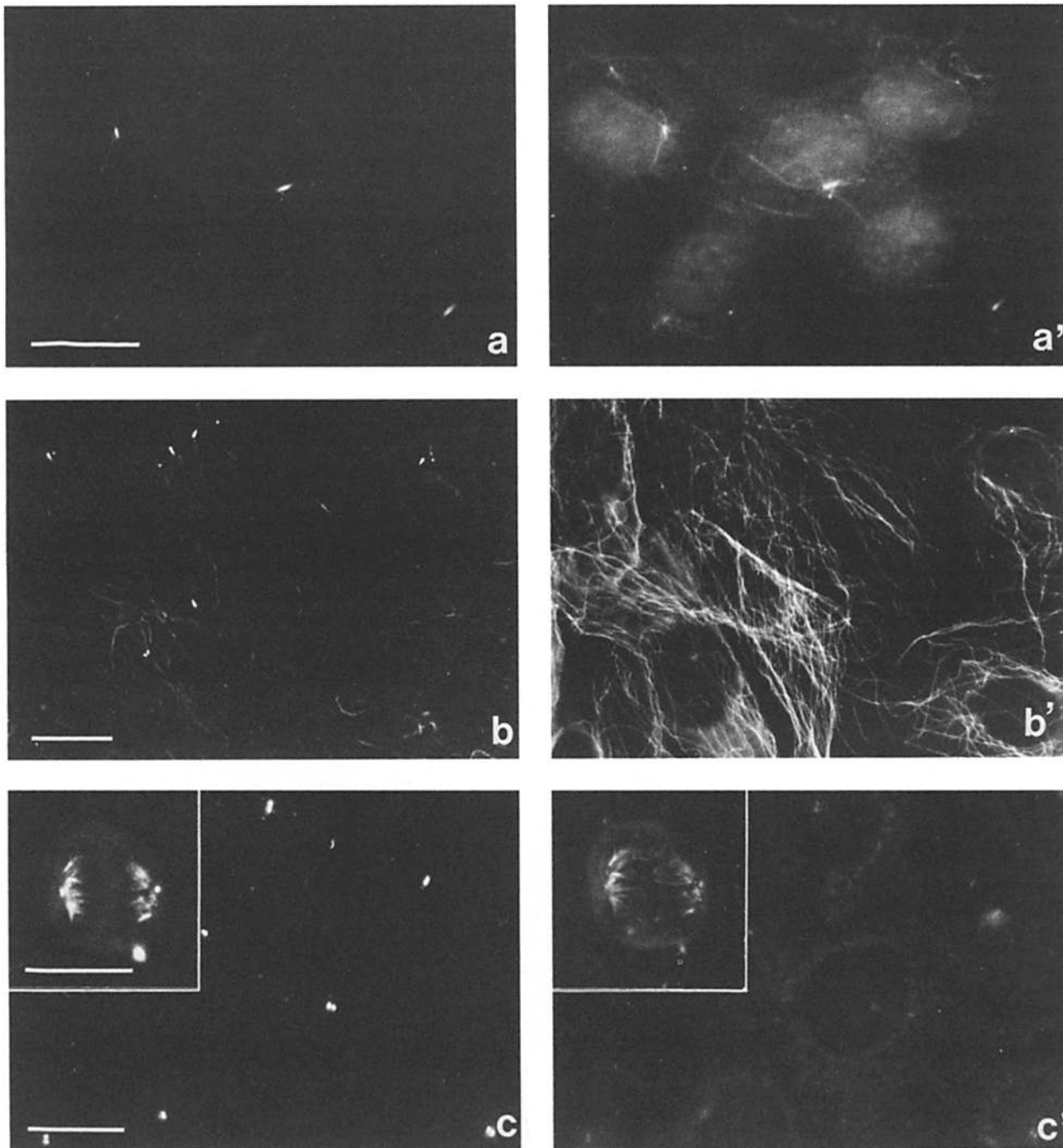


Figure 6. Anti-tubulin double-label immunofluorescence of 3T3 and HeLa cells exposed to nocodazole or 0°C. (a) 3T3 cells exposed to 1 $\mu\text{g/ml}$ nocodazole for 30 min and stained by 6-11B-1, a monoclonal antibody specific for acetylated α -tubulin. (a') Same as in a, stained by B-5-1-2, a monoclonal antibody specific for all α -tubulin isoforms. (b) 3T3 cells exposed to 0°C for 1 h and stained by 6-11B-1. (b') Same as in b, stained by B-5-1-2. (c) HeLa cells exposed to 0°C and stained by 6-11B-1. (Inset) mitotic spindle. (c') Same as in c, stained by B-5-1-2. Bars, 10 μm .

Fig. 6, b and b', shows double-label staining of 3T3 cells exposed to 0°C for 1 h and Fig. 6, c and c' shows HeLa cells treated in the same conditions. Several microtubules appear to be resistant to cold in 3T3 cells at interphase (Fig. 6 b') and in HeLa cells at mitosis, where kinetochore fibers are conserved in the spindle (inset in Fig. 6 c'). In contrast, no microtubule survived the exposure to 0°C in HeLa cells at interphase (Fig. 6 c'). Fig. 6 b shows that 3T3 cells contain acetylated α -tubulin in primary cilia, centrioles, and in few

of the cold resistant microtubules. Fig. 6 c shows that HeLa cells contain acetylated α -tubulin in kinetochore fibers (see inset) and in small paired structures that presumably are centrioles. Microtubules resistant to cold may or may not contain acetylated α -tubulin.

The Acetylation of α -Tubulin Is Reversible

The question of whether acetylated α -tubulin is present in the cells in a monomeric form of modified tubulin was inves-

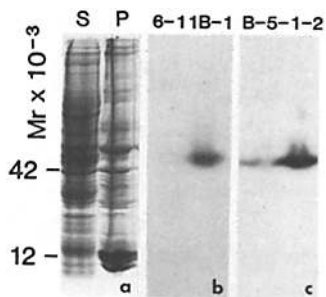


Figure 7. Antibody binding to α -tubulins present in fractions containing unpolymerized or polymerized tubulin from taxol-treated 3T3 cells. (a) Coomassie Blue-stained polypeptides after separation by SDS polyacrylamide gel electrophoresis. The position of the relative molecular mass standards is indicated at the left.

The two lanes marked *S* and *P*, respectively, contain unpolymerized and polymerized tubulin prepared as described in Materials and Methods. Two sets of the same samples were electrophoresed in parallel and then transferred to nitrocellulose. (b) Autoradiogram of the nitrocellulose incubated with 6-11B-1, a monoclonal antibody specific for acetylated α -tubulin. (c) Autoradiogram of the nitrocellulose incubated with B-5-1-2, a monoclonal antibody specific for all α -tubulin isoforms.

tigated by qualitative experiments of binding of either of the two antibodies, 6-11B-1 or B-5-1-2. The binding was tested against fractions of protein containing soluble tubulin or microtubules that were prepared from taxol-treated or untreated 3T3 cells, as described in Materials and Methods.

Fig. 7 *a* shows electrophoretograms of $\sim 40 \mu\text{g}$ of protein from supernatant and pellet fraction. The electrophoreto-

grams are composed of a large number of unresolved bands but they are different. Fig. 7, *b* and *c* shows immunoblots of the same samples incubated with the antibody 6-11B-1 or B-5-1-2. The antibody B-5-1-2 identifies one band of α -tubulin in both lanes, but the intensity of the bands indicates that the concentration of soluble α -tubulin is lower than that of polymerized α -tubulin (Fig. 7 *c*). The antibody 6-11B-1 only detects a large amount of acetylated α -tubulin in the fraction containing microtubules (Fig. 7 *b*). Supernatant fractions from untreated 3T3 cells contained more tubulin than pellet fractions, but traces of acetylated α -tubulin were detected only in the latter (not shown). These results indicate that acetylated α -tubulin in taxol-treated or untreated cells is present mainly or exclusively in polymeric form.

3T3 cells treated with $1 \mu\text{g/ml}$ of nocodazole for 30 min and HeLa cells exposed to 0°C for 1 h do not have a cytoskeleton of microtubules (see Fig. 6, *a'* and *c'*). They maintain microtubules only in centrosomes and primary cilia and rapidly form a cytoplasmic system of microtubules upon restoration of normal conditions of culture (2). Since the acetylation of α -tubulin may occur primarily or exclusively on assembled tubulin, we had the opportunity of observing where and when the acetylation is detected during the recovery from exposure to nocodazole or cold.

Assembly of cytoplasmic microtubules starts in the region of the centrosphere, which is marked by the presence of cen-

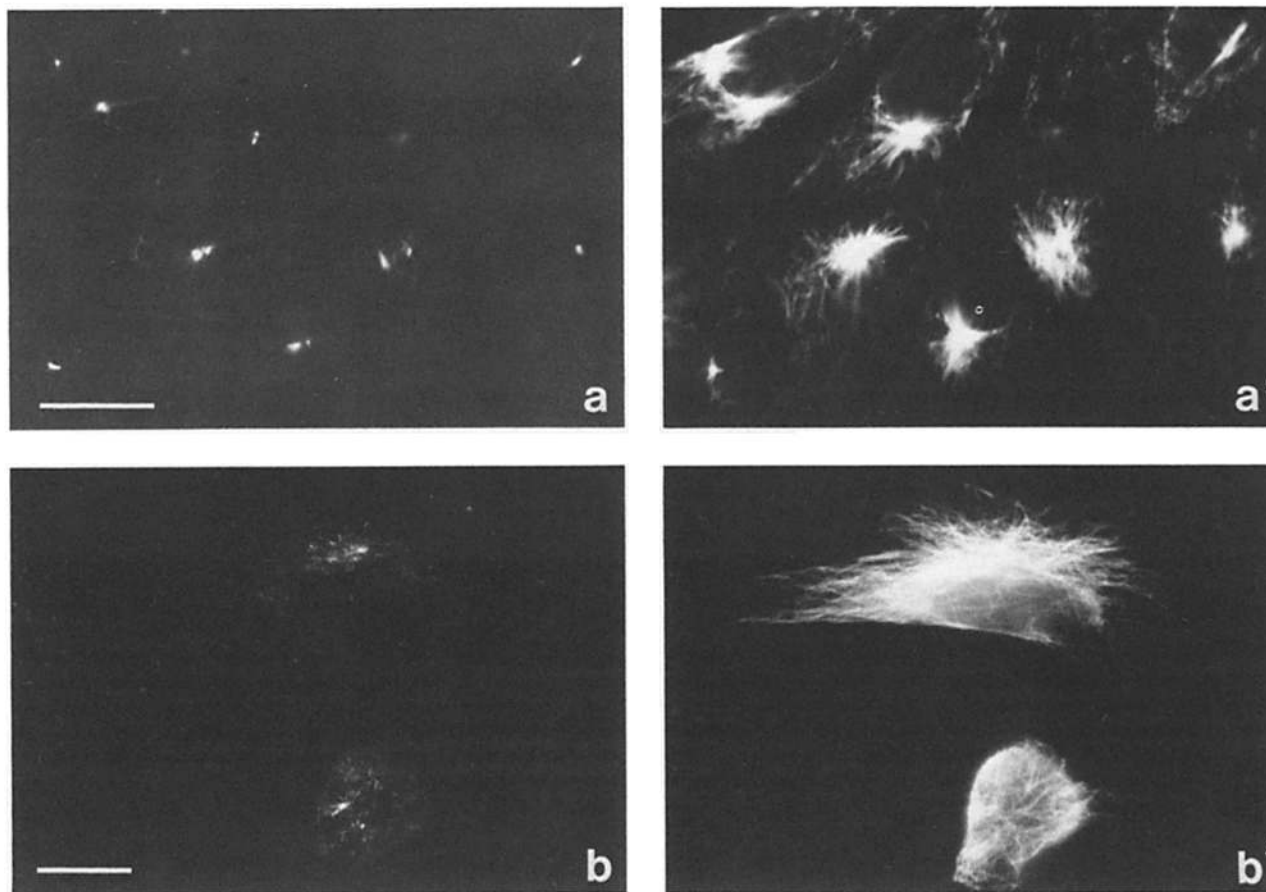


Figure 8. Anti-tubulin double-label immunofluorescence of 3T3 cells recovering from exposure to $1 \mu\text{g/ml}$ nocodazole for 30 min. (a) Cells after 90 s recovery stained by 6-11B-1, a monoclonal antibody specific for acetylated α -tubulin. (a') Same as in *a*, stained by B-5-1-2, a monoclonal antibody specific for all α -tubulin isoforms. (b) Cells after 3 min recovery stained by 6-11B-1. (b') Same as in *b*, stained by B-5-1-2. Bars, $10 \mu\text{m}$.

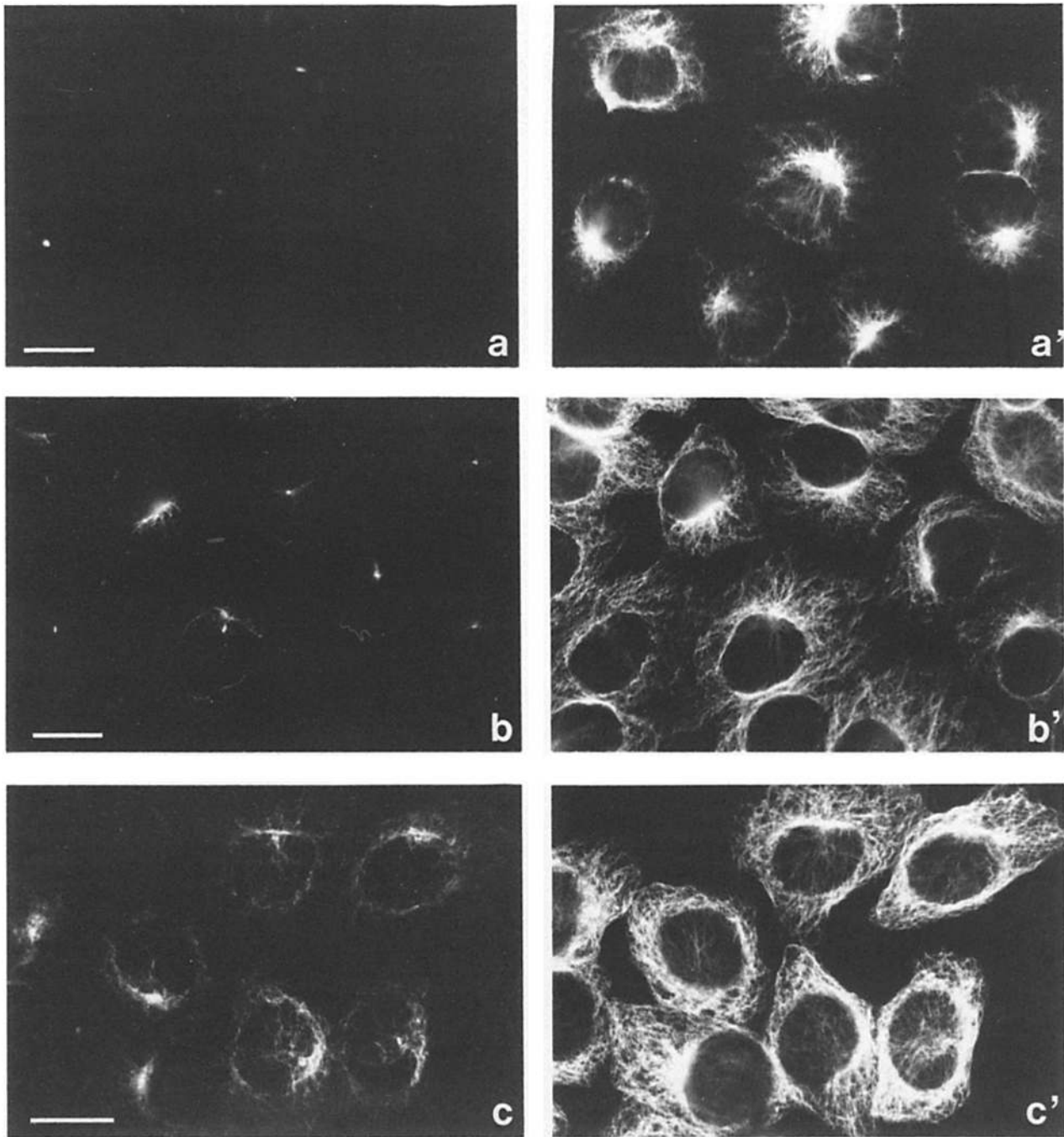


Figure 9. Anti-tubulin double-label immunofluorescence of HeLa cells recovering from exposure to 0°C for 1 h. (a) Cells after 5 min of culture at 37°C stained by 6-11B-1, a monoclonal antibody specific for acetylated α -tubulin. (a') Same as in a, stained by B-5-1-2, a monoclonal antibody specific for all α -tubulin isoforms. (b) Cells after 10 min and (c) cells after 15 min recovery, same as in a. (b' and c') Same as in b and c, stained by B-5-1-2. Bars, 10 μ m.

trioles and primary cilia. These structures already contain acetylated α -tubulin and are labeled by both antibodies 6-11B-1 and B-5-1-2. Fig. 8, a and a' shows 3T3 cells recovering under normal conditions of culture 90 s after treatment with 1 μ g/ml nocodazole for 30 min. Aster-like structures of microtubules are stained by the antibody recognizing all α -tubulins (Fig. 8 a'), whereas only centrioles and primary cilia are stained by the antibody specific for acetylated α -tubulin (Fig. 8 a). After 3 min of recovery the cells have a normal cytoskeleton of microtubules (Fig. 8 b') that contains

acetylated α -tubulin only in short segments of microtubules, primarily but not exclusively in the centrosphere region (Fig. 8 b). After 30 min of recovery the cells were indistinguishable from normal interphase cells, like those shown in Fig. 1, a and a'.

Similar patterns of staining were obtained with HeLa cells recovering from exposure to 0°C for 1 h. Cells were analyzed after 5, 10, and 15 min of culture at 37°C, as shown respectively in Fig. 9, a, b, and c, and a', b', and c'. Microtubules containing acetylated α -tubulin are barely visible in

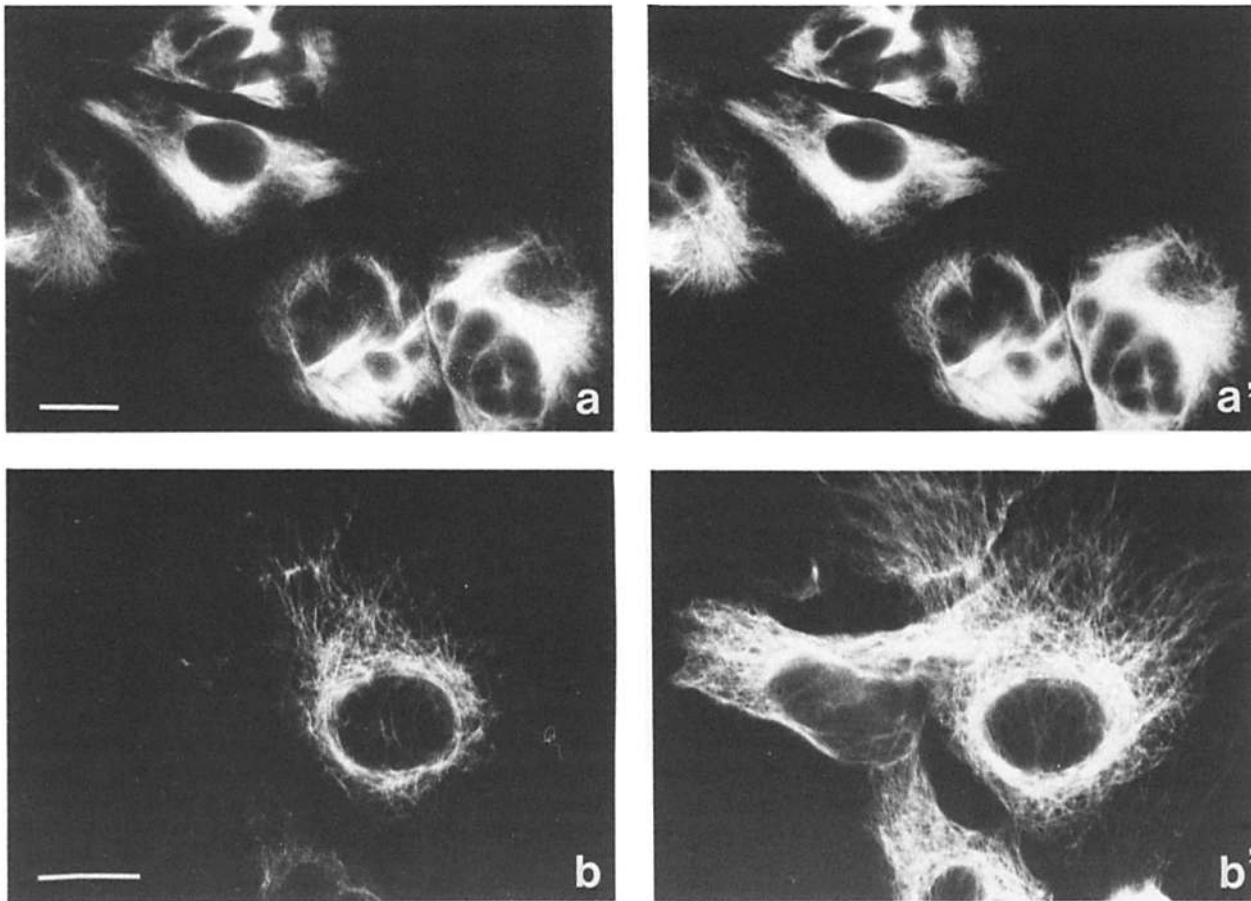


Figure 10. Anti-tubulin double-label immunofluorescence of 3T3 cells exposed overnight to 10 μM taxol. (a) Cells stained by 6-11B-1, a monoclonal antibody specific for acetylated α -tubulin. (a') Same as in a, stained by B-5-1-2, a monoclonal antibody specific for all α -tubulin isoforms. (b) Cells after 1 h recovery from taxol exposure stained by 6-11B-1. (b') Same as in b, stained by B-5-1-2. Bars, 10 μm .

the centrosphere region after 10 min of recovery (Fig. 9 b), whereas the majority of cytoplasmic microtubules are already assembled (Fig. 9 b'). After 15 min of recovery, short microtubules containing acetylated α -tubulin are seen everywhere in the cells but primarily in the centrosphere region (Fig. 9 c), whereas the antibody B-5-1-2 shows a normal cytoskeleton of microtubules (Fig. 9 c'). Acetylation of assembled α -tubulin as detected by immunofluorescence microscopy lags behind the polymerization of nearly all microtubules.

The deacetylation of α -tubulin was observed in 3T3 cells after 1 h of recovery from overnight exposure to 10 μM taxol. Fig. 10, a and a' shows 3T3 cells after drug treatment and Fig. 10, b and b' shows cells from the same culture after 1 h of recovery in normal medium. The bundles of microtubules that are formed in the presence of taxol, and the majority of acetylated α -tubulin in polymerized form, disappears during recovery. Cells in Fig. 10 b' have microtubules that appear in normal configuration and that contain low or undetectable amounts of acetylated α -tubulin (Fig. 10 b).

The amounts of tubulin in 3T3 cells recovering from taxol was also measured by dot assay. The results are reported in Table I. After 1 h of recovery, the concentration of acetylated α -tubulin returned to its pretreatment value (67% decrease), while the concentration of α -tubulin decreased by 20%. In an independent experiment, the amount of acetylated α -tu-

bulin was found to decrease by 51% after 30 min and by 68% after 1 h of recovery. The decrease in the amount of tubulin was 8 and 15%, respectively. Therefore, during recovery, the deacetylation of the α -tubulin subunits occurs faster than the degradation of tubulin.

Assembly or disassembly and acetylation or deacetylation of microtubules, as observed by immunofluorescence microscopy, were not altered if the cells were treated with 10 $\mu\text{g/ml}$ cycloheximide during exposure and recovery in every condition described above.

Butyric acid, an inhibitor of histone deacetylases (1), was added to culture medium of 3T3 cells at the concentration of 5 mM during experiments of recovery from taxol exposure. The deacetylation of α -tubulin was not inhibited.

In Vitro Acetylated α -Tubulin of PtK₂ Cells Binds the Antibody 6-11B-1

Double-label immunofluorescence of PtK₂ cells showed that antibody 6-11B-1, which is specific for acetylated α -tubulin, does not bind any microtubule structure. The antigenic determinant recognized by antibody 6-11B-1 is absent from the α -tubulin either because the acetylation of the accepting lysine(s) does not occur or because the antibody 6-11B-1 does not recognize acetylated α -tubulin from PtK₂ cells. Alternatively, the antigenic determinants may be masked.

Chemical acetylation of all the proteins contained in crude

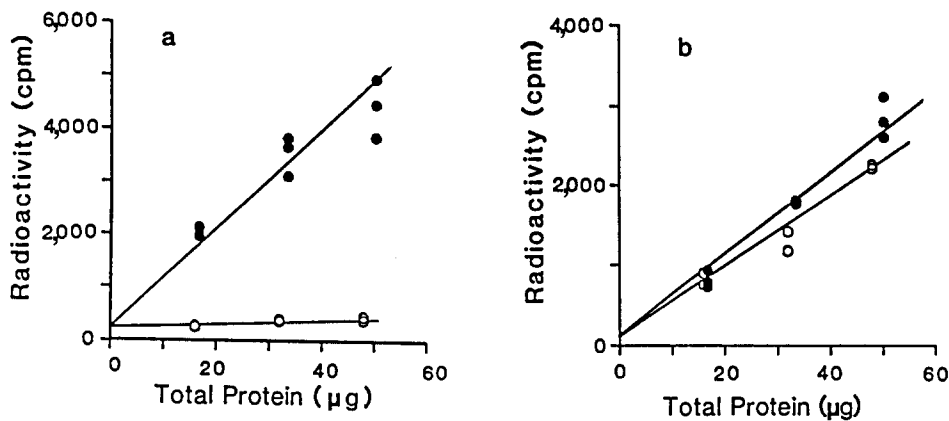


Figure 11. Antibody binding to proteins of PtK₂ cells acetylated by acetic anhydride, as measured by a quantitative dot assay. (a) Binding curves of the antibody 6-11B-1 obtained by varying amounts of cellular proteins. Triplicate samples containing modified or unmodified protein were analyzed. (Solid circles) Acetylated proteins. (Open circles) Unmodified proteins. (b) Same as in a, binding the antibody B-5-1-2.

extracts of PtK₂ cells was performed using acetic anhydride, as described in Materials and Methods. Modified and unmodified products were analyzed by immunoblots of electrophoretograms and by a dot immunoassay. Although antibody 6-11B-1 did not bind to any protein in the unmodified extract, it did recognize one polypeptide in the acetylated extract. This polypeptide had the same electrophoretic mobility as the acetylated α -tubulin (not shown). Modified and unmodified PtK₂ extracts contained approximately the same amount of α -tubulin, as shown by binding of the antibody B-5-1-2 to increasing amounts of cellular proteins (Fig. 11 b). However, the antibody 6-11B-1 bound only to modified extracts (Fig. 11 a, solid circles). The PtK₂ α -tubulins were treated with SDS and non-ionic detergents before being exposed to the antibody. Moreover, the unmodified α -tubulin did not react with the 6-11B-1 antibody in the dot immunoassay or after SDS gel electrophoresis. Therefore, the possibility that an α -tubulin-associated protein masks the 6-11B-1 epitope seems very unlikely.

Double-label immunofluorescence of PtK₂ cells with antibodies 6-11B-1 and B-5-1-2 showed that acetylation of α -tubulin and formation of 6-11B-1 epitope can also be achieved by exposing methanol-fixed cells to acetic anhydride. This experiment is shown in Fig. 12, a and a'. After modification the microtubule structures of PtK₂ cells stained by antibody 6-11B-1 or B-5-1-2 are indistinguishable. Control experiments of chemical acetylation of microtubules in situ were performed in 3T3 cells. After exposure to acetic anhydride the epitope recognized by the antibody 6-11B-1 was found in all microtubules of these cells (not shown).

The α -tubulins of PtK₂ and 3T3 cells, which under normal conditions do not bind the antibody 6-11B-1, acquire one or more epitopes after chemical acetylation of the ϵ -amino groups of all their lysines. Therefore, the lack of binding of the antibody 6-11B-1 to all microtubules of PtK₂ cells and to the majority of microtubules of 3T3 cells is due to the lack of acetylation of the α -tubulin subunits in at least one site.

Discussion

Experimental Approach

This study has two goals: to determine which set of microtubules contains an acetylated form of α -tubulin in mammalian cells in culture and to investigate whether a relationship exists between the acetylation of α -tubulin and the regulation of microtubule assembly. The experimental work has been

based on the use of one monoclonal antibody, 6-11B-1, specific for acetylated α -tubulin. The specificity of the antibody was determined by experiments binding antibodies to α -tubulin from various sources that were described in a recent paper (12). The antibody 6-11B-1 does not bind to acetylated lysine alone but recognizes an epitope present only in acetylated α -tubulin. Whether the antibody recognizes more than one epitope in the α -tubulin structure and whether it binds different forms of acetylated α -tubulin remains to be investigated.

We have used the antibody 6-11B-1 to perform immunofluorescence microscopy of methanol-fixed microtubules and binding assays to polypeptides electrotransferred or spotted on nitrocellulose sheets. The presentation of the antigenic determinant to the antibody may have been slightly different in each application, depending on the degree of denaturation of the α -tubulin. Therefore the binding activity of the antibody to the epitope could vary in each case. However, we have used the antibody in amounts spanning two orders of magnitude and against quantities of antigens varying ten times without noticing any change in the specificity of the binding activity. We have also acetylated in vitro α -tubulin together with many other polypeptides from 3T3 cells and found that after the acetylation the antibody 6-11B-1 only binds to acetylated α -tubulin. From these observations we concluded that the binding of the antibody 6-11B-1 to tubulin of mammalian cells indicates the presence of acetylated α -tubulin.

We have been able to detect by immunofluorescence microscopy specific microtubule frameworks and short segments of cytoplasmic microtubules binding the antibody 6-11B-1 in 3T3 and HeLa cells. Therefore the binding of the antibody 6-11B-1 reveals a discrete distribution of acetylated α -tubulin in different microtubule structures and in single microtubules. The possibilities that unstained microtubules are not accessible to the antibody 6-11B-1, or that the antibody B-5-1-2 competes against the binding of the antibody 6-11B-1, were ruled out. In fact, binding assay to protein fractions from 3T3 and HeLa cells indicated that only a small percentage of α -tubulin is acetylated. Moreover, all microtubules are stained by both antibodies if the α -tubulin is acetylated in vitro by acetic anhydride or in vivo in the presence of taxol. On the other hand, a lack of sensitivity of our detection system may have caused the incomplete recording of microtubule patterns containing acetylated α -tubulin.

In addition to the immunofluorescence microscopy that revealed the distribution of acetylated α -tubulin in the microtu-

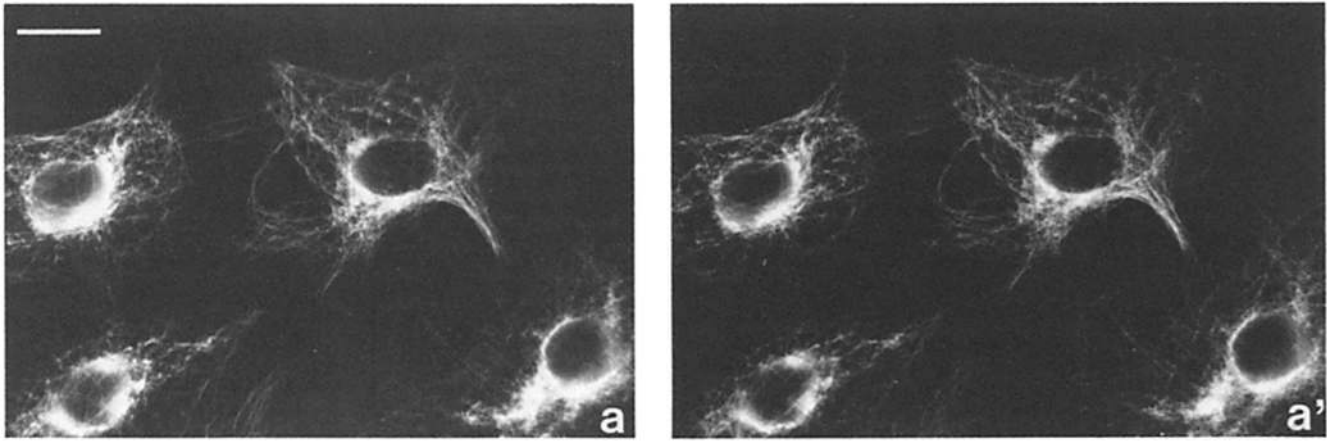


Figure 12. Anti-tubulin double-label immunofluorescence of PtK₂ cells after chemical acetylation by acetic anhydride. (a) Cells stained by 6-II-B-1. (a') Cells stained by B-5-1-2. Bar, 10 μ m.

bules, immunoblots of electrophoretograms or dot binding assays were used to detect the total content of the modified subunit in protein preparations. Therefore, we could investigate by these last methods whether the increase of microtubules containing acetylated α -tubulin in the presence of taxol was due to the induction of acetylated α -tubulin or to new assembly of preexisting modified subunits. Moreover, we could exclude the hypothesis that large amounts of unpolymerized acetylated α -tubulin are in equilibrium with microtubules containing the modified subunit. On the other hand, the absence or the presence of small amounts of modified subunits could not be evaluated because the detection procedure of antibody binding to dots or blots is sensitive to a minimum of 0.1 μ g of acetylated α -tubulin (12). Protein samples much higher than 100 μ g could not be processed by our methods.

The Acetylation of α -Tubulin Occurs on Stable Microtubules

Acetylated α -tubulin was detected in microtubule frameworks such as axonemes, basal bodies (12, 8), primary cilia, midbodies, and centrioles. Microtubules forming specific structures differ from single cytoplasmic microtubules in two properties. They have a specific position in the cell in relation to other microtubules or other cellular structures, and they are more stable under conditions of promoting disassembly of microtubules. Cytoplasmic single microtubules that contain acetylated α -tubulin appear to make an exception to this model. However, the exception may be only apparent if order and stability exist in acetylated cytoplasmic microtubules and have not been recognized. This hypothesis is supported by the results of experiments performed on cells exposed to taxol. When order and stability among all cytoplasmic microtubules is created in the presence of taxol, the acetylation of α -tubulin is extended to the whole microtubule population.

A proof of the existence of cytoplasmic microtubules that are more stable and contain acetylated α -tubulin was obtained by exposing different cells to antimicrotubule drugs that depolymerize microtubules. Drug-resistant acetylated microtubules were first observed in *Chlamydomonas* cell bodies (8). The same kinds of microtubules were found in HeLa and 3T3 cells after exposure to colchicine or nocodazole in

concentrations that do not cause side effects in tissue culture cells (2). Therefore, the resistance of cytoplasmic microtubules containing acetylated α -tubulin to the depolymerizing action of nocodazole or colchicine appears to be intrinsic to their structure. Interestingly, a population of quasi-stable microtubules has been observed in interphase cells in culture that were microinjected with labeled tubulin in the absence of antimicrotubule drugs (14). The presence of acetylated α -tubulin in these stable microtubules remains to be tested.

The resistance of microtubules to depolymerization may involve two steps. A first event causing the stabilization of microtubules could occur by end- or lateral-interaction between microtubules and other cellular entities. In a second step, the stabilization could be followed by acetylation of the α -tubulin present in the microtubule structures. The stabilization and the acetylation of microtubules obtained in the presence of taxol suggest the occurrence of these separate events. However, in vitro the stabilization of microtubules with taxol does not involve the acetylation of α -tubulin (12).

Acetylation and Deacetylation of Microtubules

The stability of cytoplasmic single microtubules must be transient since the cytoskeleton of microtubules is formed and destroyed in a continuous change of organization during the cell cycle (2). Microtubules, instead of exchanging directly with a soluble pool of modified tubulin, may be acetylated after assembly and deacetylated before disassembly. The acetylation of α -tubulin appears to occur after microtubule assembly. Suggestive evidence of this phenomenon was obtained with 3T3 cells exposed to nocodazole or HeLa cells exposed to 0°C during recovery from exposure. The appearance of acetylated α -tubulin, as observed by immunofluorescence microscopy, lagged behind the polymerization of the majority of microtubules. A second point suggesting that the acetylation of α -tubulin occurs post-assembly derives from the analysis of *Chlamydomonas* flagella. There too, acetylated α -tubulin is present in axonemal microtubules and not in the pool of matrix proteins that does contain tubulin (5, 12).

The enzymes involved in the acetylation and deacetylation of α -tubulins appear to be constitutive in HeLa and 3T3 cells because acetylation and deacetylation of microtubules in these cell lines occur even when protein synthesis is in-

hibited. The acetylase and deacetylase of α -tubulin are probably different from those modifying the histones, other proteins where the acetylation of the ϵ -amino group of lysine is common (1). In fact butyric acid, an inhibitor of histone deacetylases, does not affect the deacetylation of α -tubulin in HeLa and 3T3 cells. An acetylase activity specific for α -tubulin has been detected in *Chlamydomonas* flagella (3). Finally, the epitope recognized by the antibody 6-11B-1 is absent in the histones of many cell types that were analyzed by immunoblotting of proteins.

The Acetylation of α -Tubulin Is a Common Event

All microtubules of 3T3 and HeLa cells contain acetylated α -tubulin after exposure to taxol, and nearly every eukaryotic cell analyzed so far is provided with a subset of microtubules that binds the antibody 6-11B-1. The analysis has been extended to a variety of systems and tissues, varying from the unicellular organisms *Chlamydomonas* and *Tetrahymena* to cells of the nervous system, which are a common source of tubulin for polymerization experiments. In each case, wherever microtubules form a stable structure, as for instance the subpellicular microtubules of *Trypanosoma brucei brucei*, or lie in order, as in the neurites of primary culture of spinal cord from chick embryo, all the microtubules were found to contain acetylated α -tubulin. In contrast, microtubules present in rapidly changing structures, as leading edges of 3T3 cells migrating into a wound formed by a needle in a monolayer of cells, do not bind the antibody 6-11B-1 (Piperno, G., and M. LeDizet, unpublished results). These observations indicate that the acetylation of α -tubulin is very common and confirm the presence of acetylated α -tubulin in stable and ordered arrays of microtubules.

In view of the common occurrence of acetylated α -tubulin, it is surprising that the epitope recognized by the antibody 6-11B-1 is absent in PtK₂ cells. That absence may be due to a lack of an acetylation system or to excessive activity of deacetylases. Both possibilities remain to be investigated. PtK₁, a second cell line from kidney of *Potorous tridactylis*, which was derived from a different animal, does not bind the antibody 6-11B-1 (Piperno, G., unpublished results). Therefore, the absence of the epitope apparently is not caused by an event of differentiation or mutagenesis.

On the basis of the observations described in this report, the acetylation of α -tubulin appears to occur in a variety of

microtubule structures primarily after their assembly. Microtubules probably are stabilized by interaction with unknown cellular elements, and then are acetylated on the α -tubulin. These modified microtubules, to be kept in specific intracellular spaces, may require a lower amount of energy than those subjected to continuous assembly and disassembly.

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