Monoclonal Antibodies Specific for an Acetylated Form of α-Tubulin Recognize the Antigen in Cilia and Flagella from a Variety of Organisms

GIANNI PIPERNO* and MARGARET T. FULLER*
*The Rockefeller University, New York, New York 10021; *Department of Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, Colorado 80309

ABSTRACT Seven monoclonal antibodies raised against tubulin from the axonemes of sea urchin sperm flagella recognize an acetylated form of α-tubulin present in the axoneme of a variety of organisms. The antigen was not detected among soluble, cytoplasmic α-tubulin isoforms from a variety of cells. The specificity of the antibodies was determined by in vitro acetylation of sea urchin and Chlamydomonas cytoplasmic tubulins in crude extracts. Of all the acetylated polypeptides in the extracts, only α-tubulin became antigenic. Among Chlamydomonas tubulin isoforms, the antibodies recognize only the axonemal α-tubulin isoform acetylated in vivo on the ε-amino group of lysine(s) (L'Hernault, S. W., and J. L. Rosenbaum, 1985, Biochemistry, 24:473–478). The antibodies do not recognize unmodified axonemal α-tubulin, unassembled α-tubulin present in a flagellar matrix-plus-membrane fraction, or soluble, cytoplasmic α-tubulin from Chlamydomonas cell bodies. The antigen was found in protein fractions that contained axonemal microtubules from a variety of sources, including cilia from sea urchin blastulae and Tetrahymena, sperm and testis from Drosophila, and human sperm. In contrast, the antigen was not detected in preparations of soluble, cytoplasmic tubulin, which would not have contained tubulin from stable microtubule arrays such as centrioles, from unfertilized sea urchin eggs, Drosophila embryos, and HeLa cells. Although the acetylated α-tubulin recognized by the antibodies is present in axonemes from a variety of sources and may be necessary for axoneme formation, it is not found exclusively in any one subset of morphologically distinct axonemal microtubules. The antigen was found in similar proportions in fractions from sea urchin sperm axonemes enriched for central pair or outer doublet B or outer doublet A microtubules. Therefore the acetylation of α-tubulin does not provide the mechanism that specifies the structure of any one class of axonemal microtubules. Preliminary evidence indicates that acetylated α-tubulin is not restricted to the axoneme. The antibodies described in this report may allow us to deduce the role of tubulin acetylation in the structure and function of microtubules in vivo.

Microtubules are implicated in a variety of cellular functions including mitosis, cytokinesis, intracellular transport, the maintenance of cell shape, and the formation of motile systems such as eukaryotic cilia and flagella (5). In many cases, microtubules involved in different functions are organized into morphologically different arrays. Although the structure of a variety of microtubule frameworks has been described in detail, the molecular mechanisms that specify the assembly of morphologically and functionally different microtubule arrays have yet to be determined. The diversity among microtubular structures may be generated by the association of microtubule components with accessory proteins localized in different parts of the cell (37), by the co-polymerization of different α- and β-tubulin subunits, or by a combination of these mechanisms. Structurally different tubulin subunits have been identified as the products of different genes (for review, see reference 4) and as the result of posttranslational modifications. At least three modifications of tubulin subunits...
have been described: the phosphorylation of β-tubulin from brain (6), the removal of the carboxyterminal tyrosine from α-tubulin in vertebrate tissues (3), and the acetylation of the ε-amino group of lysine(s) in α-tubulin from Chlamydomonas flagella (18).

Posttranslational modification of tubulin subunits may be required for the assembly of particular kinds of microtubule arrays. The level of β-tubulin phosphorylation increases with the assembly of axonal microtubules during neurite outgrowth in mouse neuroblastoma cells (8). Immunofluorescence staining using polyclonal antibodies to the carboxyterminal sequence of α-tubulin with and without the terminal tyrosine demonstrated that the detyrosinated form of α-tubulin is involved in the formation of distinct subpopulations of microtubules (10). Acetylation of α-tubulin has been correlated with the assembly of the flagellar axoneme in Chlamydomonas (17). This modification is reversible; axonemal α-tubulin appears to be deacetylated during resorption of flagella (19). Although axonomal tubulin of other cells appears to be posttranslationally modified (9, 20, 30), the acetylation of α-tubulin has not yet been observed in other organisms or in other microtubule structures.

This report describes the identification and use of seven monoclonal antibodies that were raised against tubulin from the axonemes of sea urchin sperm flagella and that recognize an acetylated form of α-tubulin. Our results indicate that acetylation of α-tubulin is an important feature of axoneme assembly in a variety of organisms. Tubulin acetylation may play a prevalent role in the differentiation of microtubule structure and function.

MATERIALS AND METHODS

Preparation of Hybridomas and Monoclonal Antibodies: Seven independent cell lines producing IgGs against α-tubulin from sea urchin sperm axonemes were selected from among hybridomas derived from the fusion of a myeloma with mouse spleen cells in two separate experiments. The hybridomas are listed in Table I. The mice were immunized with a 15 S complex containing the 330,000, 134,000, and 126,000-mol-wt dynein subunits from the outer arms of sea urchin sperm axonemes. The immunogen also contained tubulin subunits, a 62,000-mol-wt polypeptide, and other minor components. The characterization of the immunogen and the procedures followed to obtain hybridomas and monoclonal antibodies against axonomal proteins have been described in a recent report (25). Several hybridomas secreting monoclonal antibodies to dynein subunits and to other axonomal components were isolated from the same hybridoma populations.

Two hybridomas, 3A5 and 4A2, secreting antibodies against tubulin from Drosophila, were prepared as follows. Embryonic tubulin was purified as described below by two rounds of assembly/disassembly from staged Drosophila ORE-R embryos 11-14 h old. Female BALB/c mice were hyperimmunized by several intraperitoneal and footpad injections of purified tubulin in Freund's adjuvant. Spleen cells were then fused to the parent myeloma cell line P3X63Ag8.653 following a modification of the procedure of Galfre et al. (7) as described by Oi and Herzenberg (24). Positive hybridoma cell cultures were identified by plate assay using the immunogen as the test antigen. 3A5 was cloned three times and 4A2 was cloned twice by limiting dilution.

Cell Electrophoresis and Immunoblotting of Sea Urchin, Chlamydomonas, Tetrahymena, and Human Proteins: Electrophoresis was performed on slab gels of reduced size (6 cm long, 20 cm wide, and 0.075 cm thick). Volumes of the samples analyzed were 5–10 μl. Stacking buffer, cathodic and anodic buffers were prepared following the method of Neville (22). The stacking gel contained 3.2% acrylamide and 0.2% N,N'-methylene-bis-acrylamide. The running gel contained 5% acrylamide, 0.5% N,N'-methylene-bis-acrylamide, 25 mM Tris/glycine, pH 8.4, and 0.1% SDS (L-8750, lot 45C-0084, Sigma Chemical Co., St. Louis, MO). Electrophoresis was performed at 20 mA for 60 min.

Isoelectric focusing was performed on slab gels (14 cm long, 12 cm wide, and 0.075 cm thick). The focusing gel contained 1 ml each of pH 5.7 and 4-6
1- to 4-d-old males and either boiled in sample buffer immediately or labeled by incubation in the presence of \(^{35}S\)methionine as described by Kemphues et al. (14) before boiling. Embryonic tubulin was prepared from staged embryos in midembryonic development, 11.5-15.5 h after oviposition at 23°C. Tubulin was purified by two rounds of assembly/dissassembly in 4 M glycerol and 1 mM GTP as described by Raff et al. (28). Two-dimensional gel electrophoresis was performed with nonequilibrium pH gradients in the first dimension as described by O’Farrell et al. (23) and as modified by Waring et al. (38) but using one part pH 3.5-10 (LK8 Instruments Inc.) and four parts pH 5-6 (Serva Fine Biochemicals Inc., Garden City Park, NY) ampholines. One-dimensional SDS-PAGE was followed the procedure of Laemmli (16), except that the lower gel was 9% acrylamide and 0.52% \(N,N'\)-methylene-bis-acrylamide. Proteins separated by gel electrophoresis were transferred to nitrocellulose paper by the method of Towbin et al. (35). For antibody staining experiments, the nitrocellulose strips containing transferred proteins were preincubated for 1-2 h at room temperature in 10% fetal calf serum in phosphate-buffered saline (PBS) (3 mM KCl, 1.5 mM KH\(\text{PO}_4\), 137 mM NaCl, 4 mM Na\(\text{2HPO}_4\), pH 7.2) with 0.05% polysorbate 80 (Tween 20). Strips were incubated in primary antibody in PBS/Tween, then incubated in peroxidase-conjugated IgG fraction of goat antiserum to mouse IgG, IgM, and IgA heavy and mixed light chains (Cappel 3211-0231). Bound antibody was localized by staining with 3,3’-diaminobenzidine tetrahydrochloride. All antibody dilutions were made in fetal calf serum/PBS/Tween. Primary antibodies consisted of dilutions of supernatants from hybridoma cultures. Routine dilutions were 1:100 for 3A5 and 4A2 and 1:10 for 6-11B-1.

**Differential Solubilization of Axonemal Microtubules and Electron Microscopy:** For differential solubilization of axonemal components, a solution of 10% Sarkosyl (Na dodecyl sarcosinate, L-5125, lot 51F-0186, Sigma Chemical Co.) was added to a 1 mg/ml suspension of sea urchin sperm axonemes in 0.9 M sucrose, 0.1 M NaCl, 1 mM MgCl\(_2\), 0.2 mM EDTA, 0.2 mM phenylmethylsulfonfyl fluoride, 5 mM Tris/Cl, pH 7.0, 0.5% Nonidet P-40, to final concentrations of 0.2 and 0.4% Sarkosyl, and the suspensions were incubated at room temperature for 30 min. Soluble proteins were separated from insoluble residues by centrifugation in a Sorvall SS34 rotor at 17,000 rpm for 15 min. Outer doublet microtubules recovered from the treatment by 0.4% Sarkosyl were suspended at a protein concentration of 9 mg/ml in 0.1 mM EDTA, 0.5 mM dithiothreitol, 1 mM Tris/Cl, pH 8, and exposed to a temperature of 40°C for 4 min (34). The insoluble residue was separated by centrifugation as above. Axonemes and the 0.2 and 0.4% Sarkosyl-insoluble residues were processed for electron microscopy as described by Huang et al. (12).

Quantitative analysis of axoneme cross sections was performed on several micrographs of thin sections taken in different parts of the pellets. More than 100 cross sections were examined in each case.

**RESULTS**

**Monoclonal Antibodies to the Major a-Tubulin of Sea Urchin Sperm Axoneme Do Not Bind to the a-Tubulin of Unfertilized Sea Urchin Egg**

Seven monospecific monoclonal antibodies against the major form of \(\alpha\)-tubulin of sea urchin sperm axoneme have the following similar characteristics. They bind to the major form of \(\alpha\)-tubulin in the axoneme but do not recognize \(\alpha\)-tubulin isolated from the cytoplasm of unfertilized sea urchin egg by taxol-induced polymerization. The binding of the antibodies to the \(\alpha\)-tubulins of sperm axonemes and unfertilized eggs was analyzed by immunoblots of gel electrophoreograms and by a quantitative immunobinding assay of proteins fixed on nitrocellulose as described in Materials and Methods. A monoclonal antibody raised against embryonic \(\alpha\)-tubulin of *Drosophila*, 3A5, and a polyclonal antibody to axonemal \(\alpha\)-tubulin of *Chlamydomonas* (27), both of which cross-react with \(\alpha\)-tubulins from a wide variety of organisms, were used as positive controls.

Fig. 1A shows electrophoreograms of approximately equal amounts of protein from sperm axonemes and taxol-stabilized microtubules from sea urchin egg. The two patterns are very similar. Two major bands, the \(\alpha\) and \(\beta\)-tubulins, are widely separated in both electrophoreograms. A small number of minor components including the dynein subunit of the axoneme and the high molecular weight components of the taxol-induced microtubules (see arrows) are also visible. Fig. 1, B and C show immunoblots of the same samples incubated with, respectively, 6-11B-1, one of the antibodies to sea urchin axonemal \(\alpha\)-tubulin, and 3A5, the antibody to embryonic tubulin of *Drosophila*. Antibody 6-11B-1 binds only to the \(\alpha\)-tubulin of the axoneme and does not recognize any component of the sample of egg microtubules. In contrast, the control antibody, 3A5, binds to \(\alpha\)-tubulins that are present in both samples. The polyclonal antibody to \(\alpha\)-tubulin of *Chlamydomonas* formed the same binding pattern as the antibody 3A5 (not shown). Antibodies 6-9H-1, 3-4B-8, 4-11F-2, 4-4H-12, C-202-13, and B-37-6 were applied at the same dilution as antibody 6-11B-1 to parallel blots and showed similar binding properties. Although the staining varied in intensity with the application of different culture supernatants, each antibody recognized the \(\alpha\)-tubulin from sperm axoneme but did not bind to any component of the egg microtubule sample.

A quantitative analysis of the binding properties of each antibody was performed by the dot assay described in Materials and Methods. Fig. 2 shows that similar amounts of the antibody 3A5 bound to aliquots of axonemes and of cytoplasmic tubulin over a wide range of antibody dilution. Antibody 6-11B-1 also bound to axonemal proteins in the same range of dilution but did not appreciably recognize cytoplasmic tubulin at any antibody dilution. The binding activities of the other six antibodies are described in Table 1. All components of the set showed the same bias. They either did not bind or had very low affinity for \(\alpha\)-tubulin from taxol-induced cytoplasmic microtubules.

Sea urchin sperm axonemes are formed by at least two \(\alpha\)-tubulin isoforms, which occur in the axoneme in different amounts and can be resolved by gel isoelectric focusing (the major form of \(\alpha\)-tubulin is occasionally resolved into three components). Both the major and the minor \(\alpha\)-tubulin isoforms are detected by the antibody 3A5 in immunoblots of isoelectric focusing gels (Fig. 3A). The minor \(\alpha\)-tubulin isoform, which migrates in a more basic position (Fig. 3A, arrow), is not recognized by antibody 6-11B-1 (Fig. 3B) or by antibody 6-9H-1 (Fig. 3C).
any of the six other antibodies of the set. The major α-tubulin isoforms, which migrate in the more acidic position, are recognized by the general antibody 3A5 and all seven specific antibodies. The presence of the epitope in the major axonemal α-tubulin coincides with the migration of the protein to a more acidic position in isoelectric focusing gels.

The assembly of *Chlamydomonas* axonemes is accompanied by the acetylation of a major form of α-tubulin, which causes the modified subunit to migrate at a more acidic position in the isoelectric focusing dimension of two-dimensional gels (18, 20). To determine whether the epitope recognized by 6-11B-1 and by the other antibodies of the set could be formed by acetylation of α-tubulin in sea urchin axonemes, we investigated whether in vitro acetylation of cytoplasmic α-tubulin from sea urchin eggs would allow it to be recognized by the specific antibodies.

**Chemical Acetylation of Cytoplasmic α-Tubulin Forms the Epitope Recognized by the Antibody 6-11B-1**

Chemical acetylation of crude extracts and purified tubulin from unfertilized sea urchin eggs was performed by acetic anhydride under conditions that allow complete and specific modification of the ε-amino group of lysines. Although many polypeptide constituents of the samples appeared to be modified by the acetic anhydride, only the α-tubulin subunit was recognized by 6-11B-1 after modification.

Fig. 4, *A* and *D* show the electrophoregrams of polypeptides present in samples of crude extracts and purified tubulin from unfertilized egg before and after chemical acetylation. Many unresolved bands are visible in the patterns formed by the extracts (Fig. 4, *A*). The migration pattern of many of the polypeptides in the two electrophoregrams is different. Therefore, many polypeptides in the crude extract have been modified. As shown in Fig. 4, *D*, in vitro acetylation causes both α- and β-tubulin to migrate more slowly in SDS gels.

Immunoblots of electrophoregrams identical to those presented in Fig. 4, *A* and *D* are shown in Fig. 4, *B* and *C*, and *E* and *F*. Modified and unmodified samples were blotted in duplicate, and the nitrocellulose strips were incubated with either the general antibody 3A5 or with the specific antibody 6-11B-1. Fig. 4, *B* and *C* show the autoradiograms of the blots

### Table I. Monoclonal Antibodies to α-Tubulin of Sea Urchin Sperm Axonemes

| Hybridomas | Binding to axonemal α-tubulin | Binding to cytoplasmic α-tubulin | Ratio |
|------------|------------------------------|----------------------------------|-------|
| 6-11B-1    | 6,348                        | 71                               | 89    |
| 6-9H-1     | 5,880                        | 153                              | 38    |
| 3-4B-8     | 4,396                        | 150                              | 29    |
| 4-11F-2    | 4,298                        | 54                               | 80    |
| 4-4H-12    | 454                          | 25                               | 18    |
| C-202-13   | 2,668                        | 26                               | 102   |
| B-37-6     | 1,415                        | 86                               | 16    |
| 3A5        | 7,169                        | 5,647                            | 1.3   |

* Each antibody was tested at a dilution of 1:100 in culture medium.
* Binding activity was detected by 125I-labeled secondary antibody. Amounts of the secondary antibody were measured in counts per minute. 350 background counts were subtracted. Numbers of counts per minute are means of two determinations.
* These hybridomas were isolated from a population obtained by an independent fusion. They were also selected by a different screening method (25).
* This hybridoma secretes an antibody to embryonic α-tubulin of *Drosophila*. The antibody recognizes a variety of α-tubulins from different organisms and was used to test for the presence of α-tubulin in the samples analyzed (see Fig. 1C.).
of the extracts. The antibody 3A5 binds only to one band, corresponding to α-tubulin in both nonacyetylated and acetylated extracts (Fig. 4 B). As in the purified tubulin sample, the modified α-tubulin from extracts migrates more slowly than the unmodified α-tubulin. Although antibody 6-11B-1 does not recognize α-tubulin in the unmodified egg extract it does recognize a single component in the acetylated extract, and this polypeptide has the same mobility as the acetylated α-tubulin (Fig. 4 C). An identical result was obtained with the immunoblots of taxol-stabilized microtubule protein (Fig. 4, E and F).

Immunoblots of unmodified and modified extracts identical to those shown in Fig. 4 C were obtained with all six of the other specific antibodies listed in Table I. All of them bind to cytoplasmic α-tubulin only after it has been acetylated (not shown). Although the binding of all seven specific antibodies to the α-tubulin is indeed correlated with the modification of the tubulin subunit, we do not yet know whether each antibody recognizes the same antigenic determinant.

The Antibody 6-11B-1 Binds to Axonemal α-Tubulin of Different Organisms

Complete amino acid sequences of α-tubulin from a variety of organisms are known and show a great degree of homology. Since the existence of ε-N-acetyllysine in the structure of the major α-tubulin from Chlamydomonas flagella has been proven by chemical analysis of the modified amino acid (18), we have assayed for the presence of the epitope recognized by the antibody 6-11B-1 in α-tubulins prepared from axonemes of Chlamydomonas and other organisms. Posttranslational acetylation of α-tubulin is correlated with axone assembly in Chlamydomonas (17). It is possible that acetylation of α-tubulin during axone assembly is a general mechanism and may be essential for the formation of the axone. In this case we would expect that the epitope recognized by 6-11B-1 might be present in axonemal tubulin from many organisms but not in cytoplasmic soluble tubulin from homologous systems. We found that all axonemal tubulins tested to date, including the tubulin from human sperm, contain at least one α-subunit that is recognized by the 6-11B-1 antibody. In contrast, soluble cytoplasmic tubulins from Chlamydomonas cell bodies and flagella, Drosophila embryos, or HeLa cells are not recognized.

Three different protein samples of Chlamydomonas were assayed by immunoblotting: the axone, a crude fraction containing soluble tubulin from the cell body, and the membrane plus matrix from flagella. Fig. 5 A shows the electrophoretograms of the polypeptides contained in the three samples. The tubulin subunits are not resolved and migrate as a major band of apparent molecular weight 50,000. Parallel samples were transferred to nitrocellulose by blotting. The blots were incubated separately with antibody 3A5 (Fig. 5 B) and the antibody 6-11B-1 (Fig. 5 C). Whereas the general antibody, 3A5, binds to α-tubulin in each sample, the specific antibody, 6-11B-1, binds only to axonemal tubulin. The tubulin present in the sample of flagellar membrane and matrix proteins is not recognized by the antibody 6-11B-1, although it is derived from the same cellular compartment that contains the axone. Therefore, in Chlamydomonas flagella, the antigenic determinant is associated specifically with the form of α-tubulin assembled in the axone.

Antibody 6-11B-1 binds specifically to the acetylated form of α-tubulin in Chlamydomonas. Blots of Chlamydomonas axoneme α-tubulins resolved in isoelectric focusing gels proved that antibody 6-11B-1 bound to the major, acetylated form of α-tubulin but not to the minor, unmodified α-tubulin (not shown). Although antibody 6-11B-1 did not recognize soluble forms of α-tubulin from Chlamydomonas cell body, 6-11B-1 did recognize cytoplasmic α-tubulin of Chlamydomonas after the protein sample was acetylated in vitro, as
shown for the cytoplasmic sea urchin tubulin.

The acetylation of α-tubulin does not differentiate flagellar axonemes from ciliary axonemes. The antigen that is recognized by the antibody 6-11B-1 is present also in axonemes of cilia from sea urchin blastulac and Tetrahymena. Only the α-tubulin was recognized in immunoblots of polypeptides from both preparations (not shown).

The antibody 6-11B-1 binds to α-tubulin from Drosophila sperm, but not to soluble α-tubulin from Drosophila embryos. Fig. 6 shows the pattern of antibody binding to proteins from Drosophila sperm (S), testis (T) and assembly purified tubulin from Drosophila embryos (E). Fig. 6 A shows the electrophoresed proteins stained with Coomassie Blue. The α- and β-tubulins are widely separated. Parallel gel lanes containing identical samples were transferred to nitrocellulose and stained with antibody 3A5 (Fig. 6 B) and antibody 6-11B-1 (Fig. 6 C). The blots shown in Fig. 6, B and C were also stained with 4A2, a monoclonal antibody that recognizes the somatic form of β-tubulin (β1) but not the testis-specific β-tubulin from Drosophila. The anti-β1 antibody was included to demonstrate that the amount of embryonic tubulin applied to the gel was similar in both cases. Antibody 3A5 recognizes all known Drosophila tubulin isoforms resolved in the gel system used for these experiments (Fig. 6 B). Antibody 6-11B-1 stains α-tubulins from sperm and testis but fails to recognize α-tubulins from embryos (Fig. 6 C). All of the other specific monoclonal antibodies in the set listed in Table I were tested against testis and purified embryonic tubulin and showed the same specificity as 6-11B-1. To rule out the possibility that acetylated α-tubulin isoforms may have been selectively lost from the embryo tubulin preparations during two rounds of microtubule assembly-disassembly, the first five antibodies of the set were also tested against a high speed supernatant from a crude embryo extract. Again the antibodies failed to recognize embryonic tubulin (data not shown).

Four α-tubulin isoforms from Drosophila testis are resolved in our two-dimensional gel system (Fig. 7, A and B). Two major α-tubulin isoforms are separated in the isoelectric focusing dimension. Each major α-tubulin has a minor α-tubulin (α*), which migrates just above it in the SDS dimension. The more acidic of the two major α-tubulins is the major α-tubulin in mature, motile sperm (29). The two major α-tubulins in testis are barely resolved in the one-dimensional gel shown in Fig. 6 B, and both bind the antibody 3A5.

Antibody 6-11B-1 stains only one of the two major α-tubulin isoforms in testis (Fig. 7 C). To determine which of the testis tubulins were recognized by antibody 6-11B-1 we stained testis proteins separated by two-dimensional PAGE. Testis were cultured for 1 h in [35S]methionine and the testis proteins were separated on two-dimensional polyacrylamide gels then transferred to nitrocellulose. After antibody staining, the position of the antigen was marked with radioactive ink (arrows). Autoradiograms of the gels show the position of the antigen with respect to other testis proteins. The acid end of the gels is to the right. (A) Testis α-tubulins stained with antibody 3A5; (B) autoradiogram of A. (C) Testis α-tubulins stained with antibody 6-11B-1; (D) autoradiogram of C. The somatic (β1) and the testis-specific (β2) β-tubulin are indicated as β1 and β2, respectively; minor and major α-tubulins are indicated as α* and α.
grams show the position of the antigen with respect to the radioactively labeled testis proteins (Fig. 7, B and D). Although the two major α-tubulin isoforms are present in adult testis in roughly equal amounts, radioactive label is preferentially incorporated into the more basic form when testis are cultured in [35S]methionine for a short period. (Fig. 7, B and D; see also reference 29). The general antibody, 3A5, recognizes all four of the testis α-tubulin isoforms resolved in our gel system (Fig. 7, A). The specific antibody, 6-11B-1, recognizes only one of the two major α-tubulin isoforms plus the minor tubulin that migrates directly above it. As shown by the position of the single arrow in Fig. 7, D, 6-11B-1 recognizes the more acidic of the two major α-tubulins, the form found in sperm.

To assay for the presence of acetylated α-tubulin in tissues of vertebrates we chose two cells of human origin, human sperm and HeLa cells. Proteins from human sperm and tubulin polymerized by addition of taxol to an extract of HeLa cells were analyzed by one-dimensional gel electrophoresis and immunoblots. The antibody 6-11B-1 recognized axonemal α-tubulin from human sperm but not cytoplasmic tubulin from HeLa cells.

Fig. 8.4 shows the electrophoretograms of the two samples. Blots from parallel gel lanes were incubated, respectively, with antibody 3A5 (Fig. 8B), and antibody 6-11B-1 (Fig. 8C). Several major bands are resolved in the electrophoretogram of human sperm; however, only two major components, presumably the α- and β-tubulin subunits, are resolved in the electrophoretogram of taxol-induced microtubules from HeLa cells (Fig. 8A). The general antibody, 3A5, recognizes α-tubulin in both samples, whereas antibody 6-11B-1 binds α-tubulin only in the sample of human sperm (Fig. 8, B and C). A preparation of axonemes from human sperm was tested in similar conditions to confirm that the antigen was associated with flagellar microtubules. The antibody 6-11B-1 recognized α-tubulin in that sample but bound also to two minor components of lower molecular weight (not shown). These minor antigens were presumably produced by proteolysis of α-tubulin after the flagellum was detached from the cell body by sonicication.

**Localization of the Antigen in the Axoneme of Sea Urchin Sperm**

The framework of the axoneme is formed by three different kinds of microtubules: the single microtubules that are located in the center of the axoneme, and the A and B tubules of the peripheral doublets. These three kinds of microtubules have different stability and are associated with different appendages (11, 39). However, none of these differences appears to be specified by the presence of acetylated α-tubulin. This modified subunit was found to be distributed in the same proportion in all three kinds of axonemal microtubule.

Sea urchin sperm axonemes were subjected to a series of extraction steps. First, both of the central pair microtubules were solubilized in the presence of 0.4% Sarkosyl, then the B tubule of the outer doublet microtubules was solubilized by treatment at 40°C, as described in Materials and Methods. The central pair, the outer doublet, and the A and B microtubules all contained the antigens recognized by the antibodies 6-11B-1 and 3A5 in approximately the same ratio. There was no evidence of enrichment of the acetylated form of α-tubulin in any of the fractions.

Fig. 9, A–C show the electron micrographs of intact axonemes and of axonemes extracted by 0.2% and 0.4% Sarkosyl. The axonemes appear well preserved, and several substructures, including dynein arms, the central pair projections, and the radial spokes, are clearly distinguished (Fig. 9A). After exposure to 0.2% Sarkosyl the axonemes appear extracted, all of the projections are no longer visible, and the axonemal framework is disrupted in some cases. However, both central pair and the outer doublet microtubules appear intact in most cross-sections (Fig. 9B). After extraction with 0.4% Sarkosyl, 67% of the central pair microtubules were solubilized, but the outer doublet microtubules appear well preserved, as shown in six selected cross-sections in Fig. 9C. Therefore, the extract obtained with 0.4% Sarkosyl was enriched in tubulin depolymerized from central pair microtubules. The preferential solubilization of the B tubule from the residual doublet microtubules was performed by thermal treatment at 40°C for 4 min. The insoluble fraction was analyzed by electron microscopy in negative staining (not shown). This fraction was composed mainly of microtubule A from outer doublet tubules.

Binding of antibodies 6-11B-1 and 3A5 to the axoneme fractions described above was tested by quantitative dot immunobinding assay. At each extraction step, both the solubilized protein and the remaining unsolubilized axoneme structures were tested for antibody binding. The results of one representative experiment are listed in Table II. Although the supernatants from axonemes exposed to 0.4% Sarkosyl and axonemes exposed to 0.4% Sarkosyl and then treated at 40°C are enriched in, respectively, tubulin from the central pair and from the B tubule, they appear to contain the same amount of acetylated tubulin found in the axonemes, outer doublet, and A tubule. No significant differences were observed in the ratios of antibodies 6-11B-1 and 3A5 bound to the antigens. Therefore the α-tubulin subunits that are present in each fraction are modified by acetylation to the same extent.
FIGURE 9 Thin-section electron micrographs of axonemes isolated from Strongylocentrotus purpuratus sperm flagella. Sections through pellets of intact axonemes (A), axonemes extracted by 0.2% Sarkosyl (B), and axonemes extracted by 0.4% Sarkosyl (C). × 55,000.

TABLE II. Localization of the Epitope Recognized by Antibody 6-11B-1 in Different Fractions of the Axoneme

| % axonemes lacking central pair microtubules | Antibody 6-11B-1 bound | Antibody 3A5 bound |
|--------------------------------------------|------------------------|-------------------|
| Untreated axonemes                         | 0.56                   |                   |
| Axonemes exposed to 0.2% Sarkosyl          | 0.48                   | 0.55              |
| Axonemes exposed to 0.4% Sarkosyl          | 0.46                   | 0.51              |
| Axonemes exposed to 0.4% Sarkosyl and at 40°C for 4 min | 0.53                   | 0.53              |
|                                            | 0.46                   |                   |

*Percentage of protein solubilized in 0.2 or 0.4% Sarkosyl refers to total axonemal protein. However, percentage of protein solubilized by thermal treatment refers to protein content of axonemes extracted by 0.4% Sarkosyl.

The seven monoclonal antibodies have behaved similarly in all assays performed to date. It is possible that they recognize the same antigenic determinant. We did not have any
reason to predict the isolation of seven hybridomas secreting antibodies with such similar properties from two different fusions. The immunogen was a 15 S dynein fraction from sea urchin sperm axoneme that contained nine polypeptides, including high molecular weight dynein subunits and axonemal α- and β-tubulin isoforms (25). The initial screen may have biased the selection of positive hybridomas toward those secreting antibody against tubulin, since α- and β-tubulin are the most prevalent polypeptides in the axoneme preparations used as antigen in the screen. However, of the 17 cell lines isolated from the two fusions, none secreted antibodies against β-tubulin, and all of the anti-α-tubulins selected were specific for the acetylated isoform.

The Antigen Is Found in Axonemal Microtubules from a Variety of Sources

The α-tubulin isoform recognized by the seven monoclonal antibodies has been observed in every cell or tissue containing axonemal structures tested to date. The antigen has been detected in axonemes from organisms as phylogenetically disparate as single cell microorganisms, marine invertebrates, insects, and mammals. The antigen is present in both flagellar and ciliary axonemes, which generate different kinds of movement.

The axoneme is a complex structure made of several distinct kinds of microtubules. The central pair microtubules and the A and B tubules of outer doublets have different structures and are associated with different appendages (11). Our extraction experiments have shown that acetylated α-tubulin is present in similar proportions in the central pair microtubules and in the A and B tubules of the outer doublets. Therefore, the acetylation of α-tubulin does not appear to provide the mechanism that specifies the structure or the associated appendages of any one class of microtubules in the axoneme. Even the differential stability under extraction conditions that distinguishes each of the central pair microtubules (39) does not seem to be due to tubulin acetylation. However, the extraction experiment(s) would not have distinguished between the possibility that both central pair microtubules contain acetylated α-tubulin and the possibility that one of the central pair microtubules is made up completely of unmodified subunits and the other is acetylated twice as much as the outer doublet microtubules. Analysis of Chlamydomonas mutants that lack central pair microtubules indicates that the outer doublet microtubules of the axoneme contain both acetylated and nonacetylated α-tubulins. Two forms of α-tubulin, corresponding to the modified and unmodified isoforms, are present in the axonemes from both wild-type and mutant cells (1).

The location of the unmodified α-tubulin in the outer doublet microtubules is not known. Unmodified α-tubulin could be localized in specific parts of the axonemes, for instance in tips of the A microtubule, which extend beyond the B microtubule at the distal end of cilia and flagella. Alternatively, unmodified α-tubulin may form a co-polymer with the acetylated isoform and be found throughout the microtubules. It is possible that the modified and unmodified α-tubulin isoforms in the axoneme are in an equilibrium mediated by acetyltransferase(s) and deacetylase(s) and that the activity of these enzymes in vivo regulates the state of assembly of the axonemes. L'Hernault and Rosenbaum (17–19) have shown that acetylation occurs in Chlamydomonas flagella during flagellar assembly and is reversed during flagellar resorption. The acetyltransferase(s) and the deacetylase(s) probably are present in the same cellular compartment as the axoneme. If these enzymes are located in the flagellum they may remain active during the isolation of flagella and axonemes in our preparations.

Sea urchin, Chlamydomonas, and Drosophila axonemes all have an α-tubulin isoform that is antigenic for the seven monoclonal antibodies described in this paper and that migrates in an acidic position in isoelectric focusing gels. Polytomella, Crithidia, and Physarum also have an axonemal α-tubulin isoform with an isoelectric point more acidic than that of cytoplasmic α-tubulins (9, 20, 30). The appearance of this acidic isoform has been correlated with the posttranslational modification of α-tubulin in these organisms. This modification may also be due to acetylation. This hypothesis may be easily confirmed using the antibodies described in this report.

The Antigen Is Not Restricted to Axonemal Microtubules

The seven monoclonal antibodies did not recognize soluble, cytoplasmic α-tubulin isoforms from unfertilized sea urchin eggs, Chlamydomonas cell bodies, Drosophila embryos, and HeLa cells. However, each of these protein fractions was isolated in a way that would enrich for tubulin precursors or tubulin depolymerized from labile microtubules that disassembled after cell breakage. The first step after homogenization in each preparation was centrifugation at high speed to remove cellular debris. Stable microtubules such as those that form axonemes, centrioles, and basal bodies probably remained intact during the fractionation and therefore did not contribute any tubulin to the soluble protein fraction. Therefore, the evidence indicates that not all α-tubulin isoforms are acetylated but does not rule out the existence of acetylated α-tubulin isoforms in microtubule structures other than the axoneme.

Preliminary experiments indicate that the antigen is probably not restricted to axonemal tubulins. Antibody binding to proteins from isolated basal bodies of Chlamydomonas showed that acetylated α-tubulin is also present in that structure. The absence of axonemes from cellular fractions that contained basal bodies was tested by monoclonal antibodies against high molecular weight polypeptide V from dynecin outer arm (12) (Piperno, G., unpublished results).

Acetylated α-tubulin isoforms may also be found in nonaxonemal microtubular structures in Crithidia. The cortex of Crithidia and other kinetoplastids contains a framework of subpellicular microtubules that can be prepared in fairly pure form and are stable under isolation conditions. The microtubules of this pellicular fraction are formed in part by an α-tubulin identical to the axonemal α-tubulin of that organism (30). It is possible that posttranslational acetylation of α-tubulin is important also for the maintenance of this stable microtubule array.

The antigen recognized by the seven monoclonal antibodies is present in substantial amounts in a variety of Drosophila tissues that are thought not to contain any significant axoneme-like structures. The general antibody, 3A5, recognized two major α-tubulins present in roughly equal amounts in blots of one-dimensional gels of protein samples prepared from larval discs, brain, and salivary glands. In these experi-
ments intact tissues were solubilized in boiling sample buffer, and there was no centrifugation step. The specific antibody, 6-11B-1, recognized only one major protein band from each tissue, and the antigen migrated in the α-tubulin position, 6-11B-1 also recognized a major protein in the α-tubulin position on one-dimensional gels from adult heads, thoraces, and whole bodies from which germline tissue had been removed, either by dissection or genetically, using a grandchildless mutation (Fuller, M., unpublished results).

The unequal distribution of α-tubulins with and without the 6-11B-1 determinant in tubulin preparations from certain cell types and organelles leads us to speculate that the modification of α-tubulin by acetylation may play an important role in the specification of tubulin structure and function. It is possible that posttranslational acetylation of α-tubulin may distinguish stable microtubules organized in complex arrays from labile microtubule populations. Analysis of the distribution of the 6-11B-1 antigen in cells that contain both kinds of α-tubulin isoforms should help determine if the acetylated isoform of α-tubulin participates in structurally or functionally distinct microtubule arrays. Ultimately the antibodies described in this report may help elucidate the possible role of acetylation of α-tubulin in the regulation of tubulin function in vivo.

Gianni Piperno thanks Dr. Giorgio Vitaldi for his advice about in vitro acetylation of proteins, Elena Sphicas for the electron microscopy of axonemes, and Jerrianne Barrett and Anne Gibbons for technical assistance. Margaret Fuller thanks Drs. Michiko Watanabe and Karen Palter for helpful advice during the isolation of monoclonal antibodies, and Joan Caution for faithful technical assistance. She also thanks Dr. Anthony Mahowald for use of his population cages for staged embryo collections and Dr. Elizabeth Raff for support and useful discussions.

G. Piperno was supported by grant GM28702 from the National Institutes of Health. M. Fuller was supported by a Postdoctoral Fellowship from the Jane Coffin Childs Fund for Medical Research, National Institute of Child Health and Human Development (NICHD) grant 1-R01-HD-16739 to E. C. Raff, a Junior Faculty Research Award from the American Cancer Society, and by NICHD grant 1-R01-HD-18127 to M. Fuller.

Received for publication 20 May 1985, and in revised form 25 July 1985.

REFERENCES

1. Adams, G. M. W., B. Huang, G. Piperno, and D. J. L. Luck. 1981. Central-pair microtubular complex of Chlamydomonas flagellum: polypeptide composition as revealed by analysis of mutants. J. Cell Biol. 91:69–76.
2. Renumbered in proof.
3. Argarana, C. E., H. S. Barra, and R. Caputo. 1978. Release of [³⁵S]tyrosine from tubulin[α]³⁵S]tyrosine by brain extract. Separation of a carboxy peptide from tubulin tyrosine ligase. Mol. Cell. Biochem. 19:17–22.
4. Cleveland, D. W. 1983. The tubulins: from DNA to RNA to protein and back again. Cell 34:330–332.
5. Dustin, P. 1978. Microtubules. Springer-Verlag New York Inc. 1–452.
6. Eipper, B. A. 1972. Rat brain microtubule protein: purification and determination of covariantly bound phosphate and carbohydrate. Proc. Natl. Acad. Sci. USA 69:2783–2787.
6a Fetto-Luzzi Ames, G., and K. Nakaide. 1976. Two-dimensional gel electrophoresis of membrane proteins. Biochemistry. 15:616–623.
7. Gallego, G., S. C. Howe, C. Mülstein, G. W. Butcher, and J. C. Howard. 1977. Antibodies to major histocompatibility antigens produced by hybrid cell lines. Nature (Lond.) 266:550–552.
8. Giard, D. L., and M. W. Kirschner. 1985. A polymer-dependent increase in phosphorylation of α-tubulin accompanies differentiation of a mouse neuroblastoma cell line. J. Cell Biol. 100:764–774.
9. Green, L. L., and W. F. Dove. 1984. Tubulin proteins and RNA during the myxamoebae flagellate transformation of Physarum polycephalum. Mol. Cell. Biol. 4:1706–1711.
10. Gunderson, G. G., M. H. Kaufman, and J. C. Bulinski. 1984. Distinct populations of microtubules: tyrosinated and non-tyrosinated alpha tubulin are distributed differently in vivo. Cell 38:779–789.
11. Hepple, J. M. 1970. Subsidiary components of the flagella of Chlamydomonas reinhardtii. J. Cell Sci. 7:823–839.
12. Huang, B., G. Piperno, and D. J. L. Luck. 1979. Paralyzed flagella mutants of Chlamydomonas reinhardtii defective for axonemal doublet microtubule arms. J. Biol. Chem. 254:3091–3099.
13. Jahn, R., W. Schiebler, and P. Greengard. 1984. A quantitative dot-immunoblotting assay for tubulins using microtubulins. B. B. Mihris. Proc. Natl. Acad. Sci. USA 81:1684–1687.
14. Kemphues, K. J., R. A. Raff, T. C. Kaufman, and E. Raff. 1979. Mutation in a structural gene for a tubulin specific to testes in Drosophila melanogaster. Proc. Natl. Acad. Sci. USA 76:3991–3995.
15. Kusnahren, K. J., T. C. Kaufman, R. A. Raff, and E. C. Raff. 1982. The testis-specific B-tubulin subunit in Drosophila melanogaster has multiple functions in spermatogenesis. Cell 31:655–670.
16. Neidle, D. M. 1971. Subcellular weight determination of protein–dodecyl sulfate complexes by gel electrophoresis in a discontinuous buffer system. J. Biol. Chem. 246:6328–6334.
17. O’Farrell, P. Z., H. M. Goodman, and P. H. O’Farrell. 1977. High resolution two-dimensional electrophoresis of basic as well as acidic proteins. Cell. 12:1133–1142.
18. Os, V. T., and L. A. Herrenberg. 1980. Immunoblogulin-producing hybrid cell lines. In Selected Methods in Cellular Immunology. S. M. Shipp, editors. W. H. Freeman & Co. Publishers, San Francisco. 351–372.
19. Piperno, G. 1984. Monoclonal antibodies to dynein subunits reveal the existence of cytoplasmic antigens in sea urchin egg. J. Cell Biol. 98:1842–1850.
20. Piperno, G., B. Huang, and D. J. L. Luck. 1977. Two-dimensional analysis of flagellar proteins from wild-type and paralyzed mutants of Chlamydomonas reinhardtii. Proc. Natl. Acad. Sci. USA 74:1600–1604.
21. Piperno, G., and D. J. L. Luck. 1977. Microtubular proteins of Chlamydomonas reinhardtii. J. Biol. Chem. 252:383–391.
22. Raff, E. C., M. T. Fuller, T. C. Kaufman, K. J. Kemphues, J. E. Rudolph, and R. A. Raff. 1982. Regulation of tubulin gene expression during embryogenesis in Drosophila melanogaster. Cell 28:33–40.
23. Raff, E. C., and M. T. Fuller. 1983. Genetic analysis of microtubule function in Drosophila. In Molecular Biology of the Cytoskeleton. G. G. Borisy, D. W. Cleveland, and D. B. Murphy, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. 293–304.
24. Russell, D. G., D. Miller, and K. Gull. 1984. Tubulin heterogeneity in the trypanosome Crithidias fasciculata. Mol. Cell. Biol. 4:779–790.
25. Scholzey, J. M., B. Neighbors, J. R. McIntosh, and E. D. Salton. 1984. Isolation of microtubules and a dynein-like MgATPase from unfertilized sea urchin eggs. J. Cell. Biol. 99:616–625.
26. Silflow, C. D., R. L. Chisholm, T. W. Conner, L. P. W. Ranum, and J. Staubus. 1985. Microtubule and a dynein-like MgATPase from unfertilized sea urchin eggs. J. Cell. Biol. 99:616–625.
27. Waring, G. E., C. D. Allis, and A. P. Mahowald. 1978. Isolation of polar granules and the identification of polar granule-specific protein. J. Cell Biol. 79:389–398.
28. Vallee, R. B. 1977. Microtubular proteins of sea urchin egg. J. Cell Biol. 76:3991–3995.
29. Vallee, R. B. 1984. A taxol-dependent procedure for the isolation of microtubules and a dynein-like MgATPase from unfertilized sea urchin eggs. J. Cell Biol. 99:616–625.
30. Williams, G. B. K., C. L. Jungreis, and J. L. Rosenbaum. 1972. Chlamydomonas flagella. I. Isolation and electrophoretic analysis of microtubules, matrix, membranes, and mastigonemes. J. Cell BioL 54:507–539.
31. Waring, G. E., C. D. Allis, and A. P. Mahowald. 1978. Isolation of polar granules and the identification of polar granule-specific protein. J. Cell Biol. 76:3991–3995.
32. Williams, G. B. K., C. L. Jungreis, and J. L. Rosenbaum. 1972. Chlamydomonas flagella. I. Isolation and electrophoretic analysis of microtubules, matrix, membranes, and mastigonemes. J. Cell BioL 54:507–539.