

# Posttranslational Modification and Microtubule Stability

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**Abstract.** We have probed the relationship between tubulin posttranslational modification and microtubule stability, using a variation of the antibody-blocking technique. In human retinoblastoma cells we find that acetylated and detyrosinated microtubules represent congruent subsets of the cells' total microtubules. We also find that stable microtubules defined as those that had not undergone polymerization within 1 h after injection of biotin-tubulin were all posttranslationally modified; furthermore dynamic microtubules were all unmodified. We therefore conclude that in these cells the stable, acetylated, and detyrosinated microtubules

represent the same subset of the cells' total network. Posttranslational modification, however, is not a prerequisite for microtubule stability and vice versa. *Potorous tridactylis* kidney cells have no detectable acetylated microtubules but do have a sizable subset of stable ones, and chick embryo fibroblast cells are extensively modified but have few stable microtubules. We conclude that different cell types can create specific microtubule subsets by modulating the relative rates of posttranslational modification and microtubule turnover.

THE microtubule cytoskeleton is involved in many cellular functions, such as mitosis, morphogenesis, motility, and intracellular organelle transport. For the cell to perform some of these functions simultaneously the microtubules may have to be differentiated so that different microtubules can carry out different functions. There are several means by which the microtubules of a cell can be differentiated. Among these are differential binding of proteins, specific incorporation of tubulin isotypes, and posttranslational modification of tubulin. Although differential binding of microtubule associated proteins has been shown to occur in axons and dendrites in neuronal cells (Binder et al., 1986; Matus et al., 1986), it has not yet been reported to occur in nonneuronal cells. Incorporation of tubulin isotypes into spatially distinct microtubule subsets is also possible, but in one case where it has been well studied, the testis-specific isotype in *Drosophila*, the cell-specific isotype is used for all common cytoskeletal functions as well for tissue-specific functions (Kemphues et al., 1982). Recent studies in mammals also suggest no spatial differentiation of isotypes (Cowan, N.J., personal communication; Asai, D. J., unpublished observations). Two groups have, however, reported the existence of antigenically and morphologically distinct microtubule subsets in the same cell. The first group used a monoclonal antibody, subsequently shown to recognize acetylated  $\alpha$ -tubulin, to reveal a distinct subset of microtubules in mouse and human fibroblasts (Thompson et al., 1984; Thompson, W. C., personal communication). These microtubules, representing between 10 and 40% of the cells' microtubules appeared to be more curly and wavy than the

majority of the cells' microtubules, and were concentrated near the cell center. The second group used a polyclonal antibody which they raised to distinguish detyrosinated tubulin (named Glu tubulin for its COOH-terminal amino acid) from tyrosinated tubulin (named tyr tubulin) to reveal a distinct set of microtubules in African green monkey kidney cells that appeared similar in distribution to the acetylated microtubules in other cells (Gundersen et al., 1984).

Recently, using a new immunocytochemical technique in conjunction with microinjection of biotin-tubulin, we identified another subset of microtubules by the functional criterion of stability to exchange with free intracellular tubulin over long periods of time (Schulze and Kirschner, 1987). These kinetically differentiated microtubules represent approximately 10–20% of a cell's microtubules and have half-lives of  $\sim 1$  h, as opposed to 5–10 min for the majority of the cells' microtubules. In addition to their kinetic distinction these microtubules are morphologically distinct. Like the acetylated and Glu microtubules they are more curly and wavy, located preferentially near the cell center, and rarely extend to the cell periphery.

Because of the morphologic similarity of these three microtubule subsets, and because one might imagine that increased stability is related to increased modification, we thought these subsets might be related causally. Using variations of the antibody blocking technique in conjunction with the use of antibodies specific for posttranslationally modified Glu and acetylated tubulin, we have been able to probe directly the relationship among these three subsets. We find that in human retinoblastoma cells there seems to be a pre-

cise correspondence between the three subsets on the single microtubule level. This does not mean that there is always an obligate causal connection, because in another cell line where there are no acetylated microtubules there are still extensive arrays of both deetyrosinated and stable microtubules. Similarly in another cell line there is both deetyrosination and some acetylation but few stable microtubules. In no cells that we have examined does the dynamic microtubule population preferentially accumulate posttranslational modifications. We conclude from these studies that posttranslational modification can be, but is not always correlated with microtubule stability. Cells apparently can modulate their rates of posttranslational modification and microtubule turnover independently to create spatially, and temporally distinct microtubule subsets.

## Materials and Methods

### Materials

*N*-hydroxysuccinimidyl biotin was from Polysciences, Inc. (Warrington, PA); rabbit anti-biotin antibody was from Enzo Biochem, Inc. (New York, NY). Mouse monoclonal anti- $\beta$ -tubulin was a kind gift of Dr. S. H. Bose (Protein Databases, Inc., Huntington Station, NY). Mouse monoclonal anti-acetylated-tubulin was raised as previously described (Thompson et al., 1984). Rabbit polyclonal anti-deetyrosinated-tubulin (anti-Glu) was raised as previously described (Gundersen et al., 1984). Unlabeled, and rhodamine- and fluorescein-labeled secondary antibodies were from Cappel Laboratories (Cooper Biomedical Inc., Malvern, PA). Texas Red-labeled and unlabeled rat anti-mouse and mouse anti-rat antibodies were from Accurate Chemical and Scientific Corporation (Westbury, NY).

### Tubulin Preparation

Phosphocellulose-purified tubulin was prepared from bovine brains by a modification of the procedure of Weingarten et al. (1975) as described in Mitchison and Kirschner (1984). Sea urchin tubulin was prepared by the method of Suprenant and Marsh (1987). Microtubule protein was chemically acetylated after the method of Piperno and Fuller (1985). Protein concentrations were determined by the method of Bradford (1976), using bovine serum albumin as a standard.

### Preparation of Biotinylated Tubulin

Biotinylated tubulin was prepared as described in Kristofferson et al. (1986) and Mitchison and Kirschner (1985) with modifications for microinjection as in Schulze and Kirschner (1986).

### Cell Culture

African Green monkey kidney fibroblasts (BSC1) (a kind gift of U. Euteneuer, University of California at Berkeley) were grown in Dulbecco's modified Eagle's medium (DME) supplemented with 10% fetal calf serum (Gibco, Grand Island, NY). Human retinoblastoma cells (SKNSH)<sup>1</sup> (a kind gift of Manfred Schwab, University of California at San Francisco) were grown in RPMI 1640 with 10% fetal calf serum (Bluestein, 1978). *Potorous tridactylis* kidney (Ptk1) cells (a kind gift of J. Steinhart, University of California, Berkeley) were grown in Hams F-12 supplemented with 10% fetal calf serum. Chicken embryo fibroblasts were grown in DME-H21 supplemented with 10% fetal calf serum. For microinjection studies, the cells were trypsinized off tissue culture dishes and replated onto 1-in-diam glass coverslips. BSC1, Ptk1, and chicken embryo fibroblast cells were allowed to settle for 2 d before use; SKNSH cells were allowed to settle for 1 d, and differentiated in 30  $\mu$ g/ml retinoic acid for  $\sim$ 3 d before use.

### Microinjection

Cells on coverglasses were placed in a thermostatted microinjection cham-

ber at 37°C as described in Schulze and Kirschner (1986). The chamber was then placed on a Zeiss ICM 405 inverted microscope allowing direct access from above. The cells were then pressure-microinjected using the technique of Graessman and Graessman (1976). Microinjection needles were drawn out to  $\sim$ 0.5–1- $\mu$ m-diam tips using a micropipette puller model 720 equipped with patch clamp adapter 728A (David Kopf Instruments, Tujunga, CA). All injections were of 10 mg/ml biotin tubulin, and we estimate that approximately one-tenth of a cell volume was typically injected (Graessman et al., 1980).

### Antibody-blocking Protocols

To establish the relationship between microtubule subsets, several variations of the previously described antibody-blocking technique were used. The microinjection of cells, when necessary, was done as described above. In preparation for the antibody-blocking protocols, cells were permeabilized and fixed as described previously (Schulze and Kirschner, 1987). The various antibody-blocking protocols are very similar, the differences depending on the species of the primary antibodies whose distribution and overlap are to be compared. They are all two-step procedures, the first step is referred to as the blocking step and the second as the staining step. The blocking step is composed of two parts. In the first, cells are reacted with the primary antibody which will serve as the basis for blocking with secondary antibodies. The second part is the actual block; in this part the cells are sequentially reacted with a set of four serially reacted secondary antibodies. The specific antibodies used depend on the species of the primary antibody used in the first step. If the primary was a rabbit polyclonal, then blockage is achieved by using the following sequence of secondary antibodies: fluorescein-labeled goat anti-rabbit (40 min), fluorescein-labeled rabbit anti-goat (40 min), unlabeled goat anti-rabbit (40 min) followed by unlabeled rabbit anti-goat (40 min). This is referred to as the rabbit block (Rabbit<sup>a</sup> in Table I). If the primary was a mouse monoclonal antibody, then one sequentially incubates with Texas Red-labeled rat anti-mouse (40 min), Texas Red-labeled mouse anti-rat (40 min), unlabeled rat anti-mouse (40 min), followed by unlabeled mouse anti-rat (40 min). This is referred to as the mouse block (Mouse<sup>b</sup> in Table I). Upon completion of this first step, the microtubules that reacted with the primary antibody are masked from further reaction with further primary antitubulin antibodies. The second step in the antibody-blocking protocol is the staining of the unlabeled microtubules. In this step one reacts the remaining unblocked microtubules with a second primary antibody whose distribution and overlap are to be compared with the original set. The primary antibody used in this step cannot be the same species as that used in the blocking step due to subsequent cross-reaction with the secondary antibodies. The second primary antibody is followed by a series of two sequentially applied, fluorescently labeled secondary antibodies. If the primary used in the blocking step was a rabbit polyclonal antibody, then the primary antibody used in the staining step must be a mouse monoclonal, and this would be followed by sequential incubations with Texas Red rat anti-mouse (40 min), followed by Texas Red mouse anti-rat (40 min). We refer to this as a mouse stain (Mouse<sup>c</sup> in Table I). If the primary used in the blocking step was a mouse monoclonal antibody, then the primary antibody used in the staining step must be a rabbit polyclonal, and this would be followed by sequential incubations with fluorescein-labeled rabbit anti-goat (40 min) and fluorescein-labeled goat anti-rabbit. We refer to this as a rabbit stain (Rabbit<sup>d</sup> in Table I). Therefore, all blocking protocols consist of two steps: incubation with a primary antibody which is then blocked by a series of four secondary antibodies, followed by staining with a second primary antibody visualized by a series of two secondary antibodies. In these protocols, detailed in Table I, all antibodies are used at concentrations of 5  $\mu$ g/ml at room temperature. Between each successive incubation with antibody, the cells are washed five times with antibody buffer (PBS + 0.1% Triton X-100). Primary rabbit anti-biotin (abbreviated Bio in Table I) is used for 2 h, primary rabbit anti-Glu-tubulin (abbreviated Glu in Table I), and primary mouse anti-acetylated-tubulin (abbreviated Ac in Table I) are each used for 3–4 h. All secondary antibodies are incubated for 40 min. Protocol D (the double-block protocol, Table I) technically differs from the others by using two sequentially applied, primary antibodies in the blocking step (Glu first for 3 h followed by Bio for 1 h); it is otherwise the same as the other protocols. The specific protocols used are identified in the text and figure legends.

### Identity of Growing Microtubules

To see whether growing microtubules were acetylated and vice versa, we microinjected cells with biotin-tubulin, as previously described, and incubated the cells for 5 min or less before permeabilization and fixation in

1. Abbreviations used in this paper: Ptk1, *Potorous tridactylis* kidney (cells); SKNSH, human retinoblastoma (cells).

**Table I. Summary of Protocols**

Protocol	A	B	C	D	E
First primary	Ac	Glu	Bio	Glu/Bio	Bio
Block	Mouse <sup>b</sup>	Rabbit <sup>b</sup>	Rabbit <sup>b</sup>	Rabbit <sup>b</sup>	Rabbit <sup>b</sup>
Second primary	Glu	Ac	Ac	Tb	Tb
Stain	Rabbit <sup>a</sup>	Mouse <sup>a</sup>	Mouse <sup>a</sup>	Mouse <sup>a</sup>	Mouse <sup>a</sup>

Primary antibodies are rabbit polyclonal anti-Glu peptide (Glu), mouse monoclonal anti-acetylated tubulin (Ac), and rabbit polyclonal anti-biotin (Bio). Rabbit<sup>b</sup>, Mouse<sup>b</sup>, Rabbit<sup>a</sup>, and Mouse<sup>a</sup>, are the rabbit block, mouse block, rabbit stain, and mouse stain, respectively, as detailed in the text. The protocols are identified in Materials and Methods and in figure legends.

preparation for antibiotin, anti-acetylated-tubulin double-label immunofluorescence. The double-label immunofluorescence was carried out as described below. The cells are first incubated with mouse anti-acetylated-tubulin (3 h) followed by the mouse stain (Mouse<sup>a</sup>) as in the previous section. This stains the acetylated microtubules with Texas Red. The cells are next incubated with rabbit antibiotin (1 h), followed by the rabbit stain (Rabbit<sup>a</sup>) as above. This stains the newly grown biotin-tubulin-containing microtubule segments with fluorescein. The cells are then photographed as described below. By using two identical loupes, one can superimpose the two images on a light box and count and score the acetylated microtubules and the newly growing biotin-tubulin segments to check for growing, acetylated, microtubules as detailed in the results section.

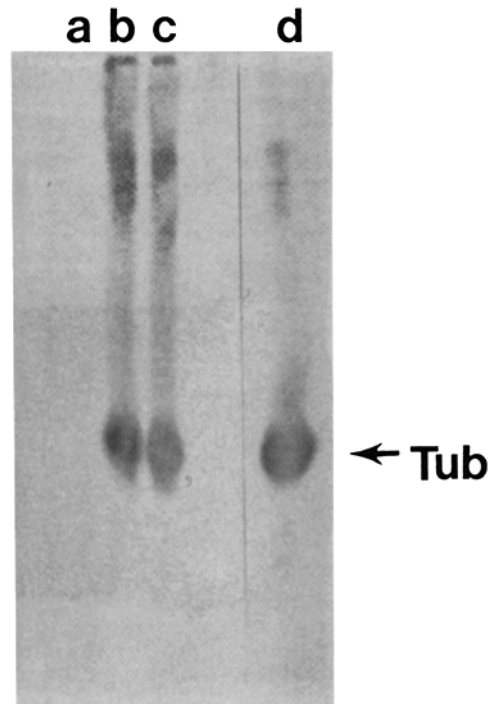
### Immunofluorescence Photomicroscopy

After preparation using any of the above described immunofluorescence protocols, the cells on coverslips are rinsed in antibody buffer, and mounted in 90% vol/vol glycerol: 20 mM Tris, pH 7.9. Microtubule networks were photographed on hypersensitized Kodak Tech-Pan (2415) film (Eastman Kodak Co., Rochester, NY). Hypersensitization was carried out in a Lumicon model 1200 hypersensitization chamber (Livermore, CA). For the Texas Red, and fluorescein filters in a Zeiss photomicroscope III, the film settings were Din 33 and 27, respectively. The film is developed in Kodak D-19 for 4 min, and fixed in Kodak rapid fix for 1 min. The use of this film aids in accurate analysis of highly magnified images and is well matched to the high resolution of the immunofluorescence techniques employed.

## Results

### Identity of Acetylated vs. Glu Microtubules

**Identity of Tubulin Classes.** We have employed antibodies with specificity for various forms of modified tubulin: A mouse monoclonal antibody to  $\beta$ -tubulin used to label all microtubule classes (Blöse et al., 1984), a polyclonal antibody to a detyrosinated peptide (Glu tubulin) and a polyclonal antibody to the tyrosinated peptide (Tyr tubulin) have also been previously characterized (Gundersen et al., 1984). We have also used a monoclonal antibody that reacts with the acetylated isoform of  $\alpha$ -tubulin. This antibody has been shown to stain a subset of microtubules (Thompson et al., 1982). As shown in Fig. 1 this antibody reacts with chemically acetylated tubulin along with tubulin purified from sea urchin embryonic cilia. It is thus similar to the antibody described by Piperno et al., (1987). Biotin-tubulin was detected by a rabbit polyclonal antibody (Mitchison and Kirschner, 1984; Schulze and Kirschner, 1986). Each antibody has its limit of detection so that, for example, where we use the term Glu microtubule, it means that enough tubulin modified by detyrosination is present to be detected by the antibody. The identity of the posttranslational modification will be used as a nomenclature for the microtubule, but it always should be borne in mind that this is an operational definition. The in-

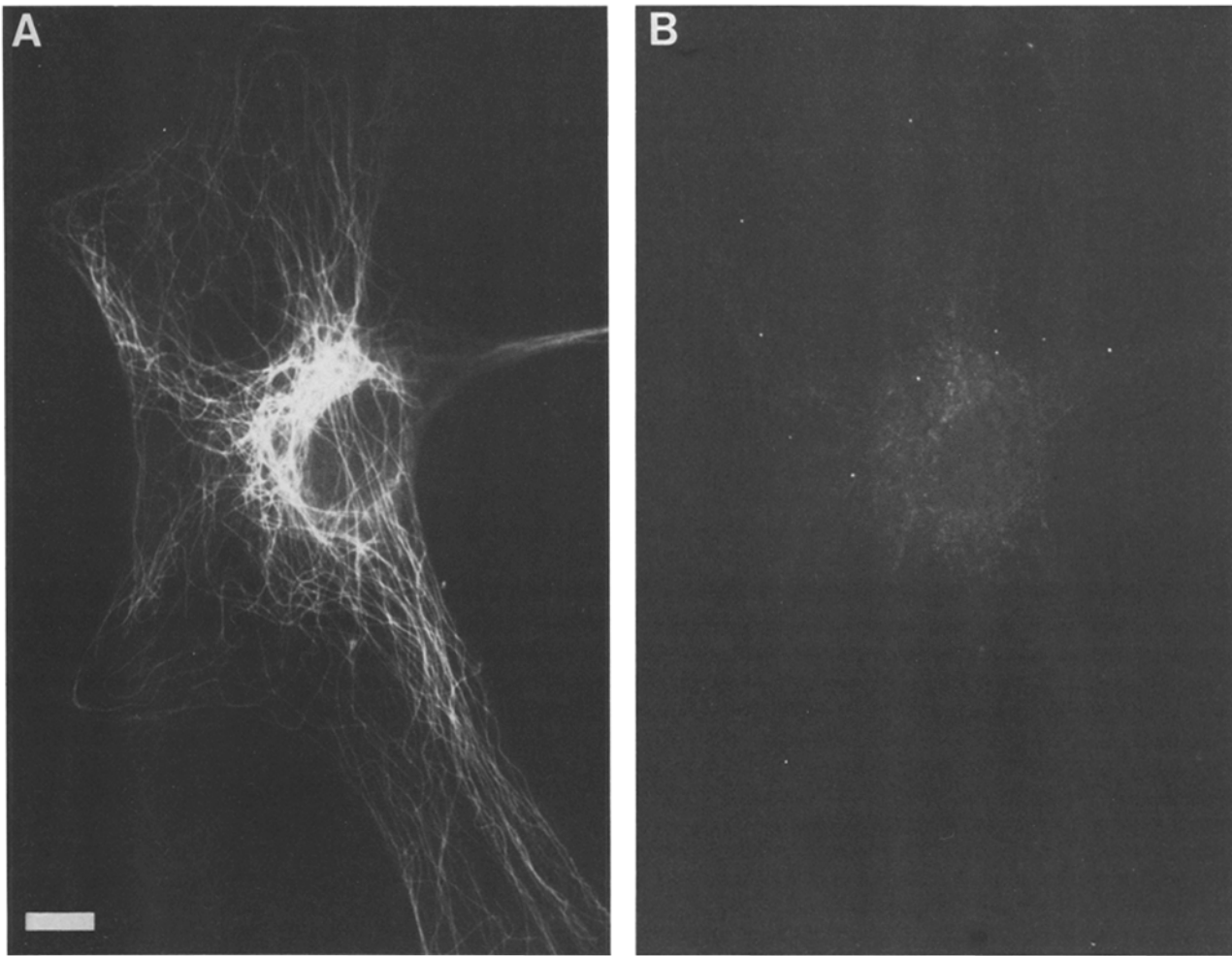


**Figure 1.** Western immunoblots of sea urchin tubulins probed with mAb 1-6.1 and developed with horseradish peroxidase. Samples were electrophoresed on a 10% acrylamide/0.14% bisacrylamide/4 M urea gel and transferred to nitrocellulose. Lane a, untreated sea urchin egg microtubule protein; lanes b and c, acetylated microtubule protein; lane d, sea urchin embryonic cilia (untreated). Equal amounts of protein were loaded in lanes a, b, and c. Comparison of the blot to the stained gel showed that mAb 1-6.1 reacted with acetylated  $\alpha$ -tubulin.

terpretation of this operational definition will be discussed more fully in the Discussion.

**There Are No Nonacetylated Glu Microtubules in SKNSH Cells.** To probe the relationship between the subsets of Glu and acetylated microtubules we initially asked whether individual microtubules were both acetylated and Glu by double-label immunofluorescence. This method proved to have only limited utility. It allowed us to conclude that, in SKNSH (human retinoblastoma) cells, a few microtubules that could be visualized in the cell periphery were both unambiguously Glu and acetylated. However, most of the post-translationally modified microtubules in cells were near the center, where there was too much overlap and insufficient resolution to detect single microtubules. Therefore characterization by double labeling was limited to the cell periphery and encompassed only a small fraction of the microtubules of the cell.

The assignment of microtubules to Glu and acetylated classes throughout the cell could be accomplished by using a variation of the recently described antibody-blocking technique. This technique had been shown previously to distinguish clearly between microtubules that had incorporated biotin-tubulin and those that had not (Schulze and Kirschner, 1987). Fig. 2 shows the results of one such experiment (antibody-blocking protocol A in Table I) designed to test whether there were any Glu microtubules that were not acetylated. Briefly, fixed and permeabilized SKNSH cells



**Figure 2.** Immunofluorescence micrograph of an SKNSH cell prepared using the antibody-blocking methodology (protocol A). (A) Anti-acetylated-tubulin staining showing the microtubules that contain acetylated tubulin. These microtubules are also masked. (B) Anti-Glu immunofluorescence showing the cells' lack of nonacetylated Glu microtubules. Bar, 10  $\mu$ m.

were reacted with the anti-acetylated-tubulin primary antibody. The cells were then reacted with a series of Texas Red-labeled secondary and tertiary antibodies that allowed the visualization of the acetylated microtubule subset (Fig. 2 A). This was followed by reaction with unlabeled quaternary and quinary antibodies. These unlabeled antibodies effectively masked the underlying acetylated microtubules from further reaction with any further primary antitubulin antibodies. To test whether, in these cells, any microtubules that were not acetylated, and therefore were not masked, were

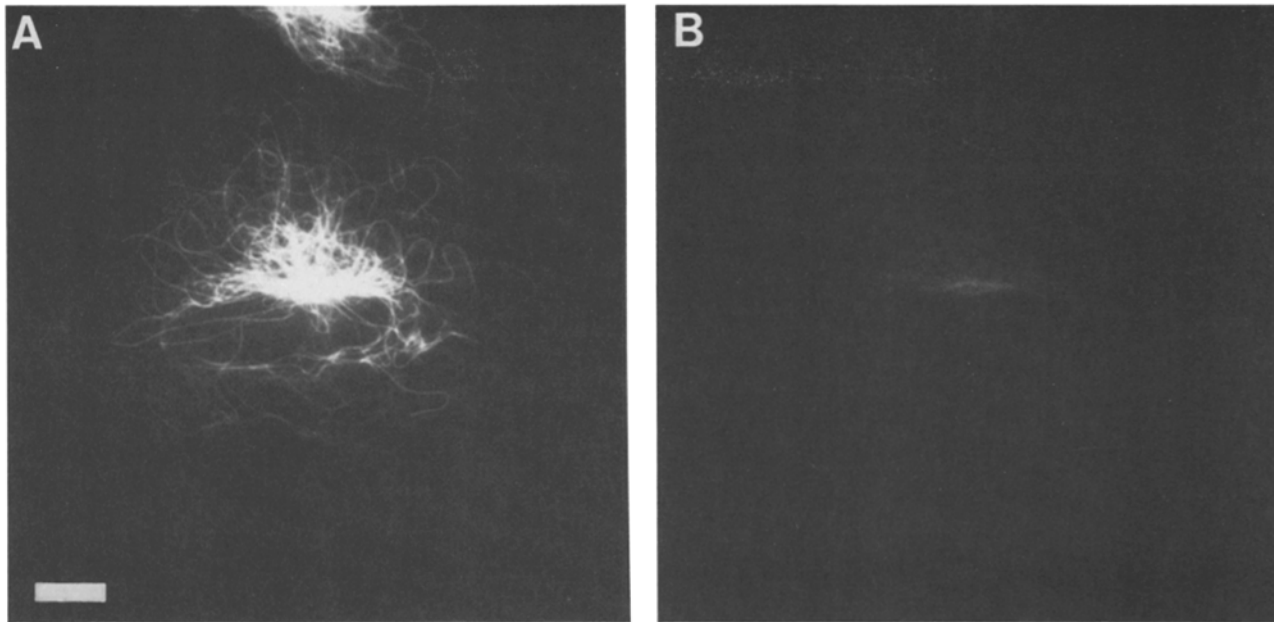
Glu, one need only stain with anti-Glu tubulin followed with the appropriate fluorescein-labeled secondary antibodies (Fig. 2 B). Clearly, in this cell, there were no Glu microtubules that were also not acetylated. Of 100 such cells examined, none had nonacetylated Glu microtubules (Table II).

**There Are No Non-Glu-Acetylated Microtubules in SKNSH Cells.** Based on the previous experiment, the Glu microtubules in SKNSH cells must be either a proper subset, or the same set as the acetylated microtubules. To determine which, we asked whether there were any microtubules that

**Table II. Microtubule Staining Scores**

Block	Stain		
	Anti-Glu tubulin	Anti-acetylated tubulin	Antitubulin
Anti-acetylated tubulin	0 (0)	—	—
Anti-Glu tubulin	—	0 (0)	4
Antibiotin tubulin	—	1.7 (0-3)*	1.9 (0-3)‡
Antibiotin and anti-Glu tubulin	—	—	0 (0)§

The number of microtubules per cell was estimated visually using the following scoring: 0, no immunofluorescent microtubules per cell; 1, five or fewer per cell; 2, ~5-20% estimated; 3, 20-50% estimated; 4, >50% estimated. The average score is shown and the range is indicated in parentheses. The number of cells counted was >100 in all cases except those indicated by a footnote. In those cases the number of cells examined was the following: \* 67 cells; ‡ 116 cells; and § 15 cells.



**Figure 3.** Antibody-blocking immunofluorescence of an SKNSH cell using protocol B. (A) Anti-Glu tubulin staining showing the masked Glu microtubules. (B) Anti-acetylated-tubulin immunofluorescence showing the cells' lack of non-Glu-acetylated microtubules. Bar, 10  $\mu$ m.

were not Glu but which were acetylated using antibody-blocking protocol B detailed in Table I. This experiment is the converse of the previous one. Fig. 3 A shows the anti-Glu tubulin staining of an SKNSH cell. As in the first experiment, these microtubules are masked from further reaction with other primary antibodies by a series of secondary antibodies. Fig. 3 B shows the anti-acetylated-tubulin staining, again revealing the null set; i.e., there are no microtubules in this cell that are not Glu, but are acetylated. Again, 100 such cells were examined, and none had any non-Glu-acetylated microtubules (see Table II). Logically, then, the acetylated and Glu microtubule subsets are the same set in this cell type.

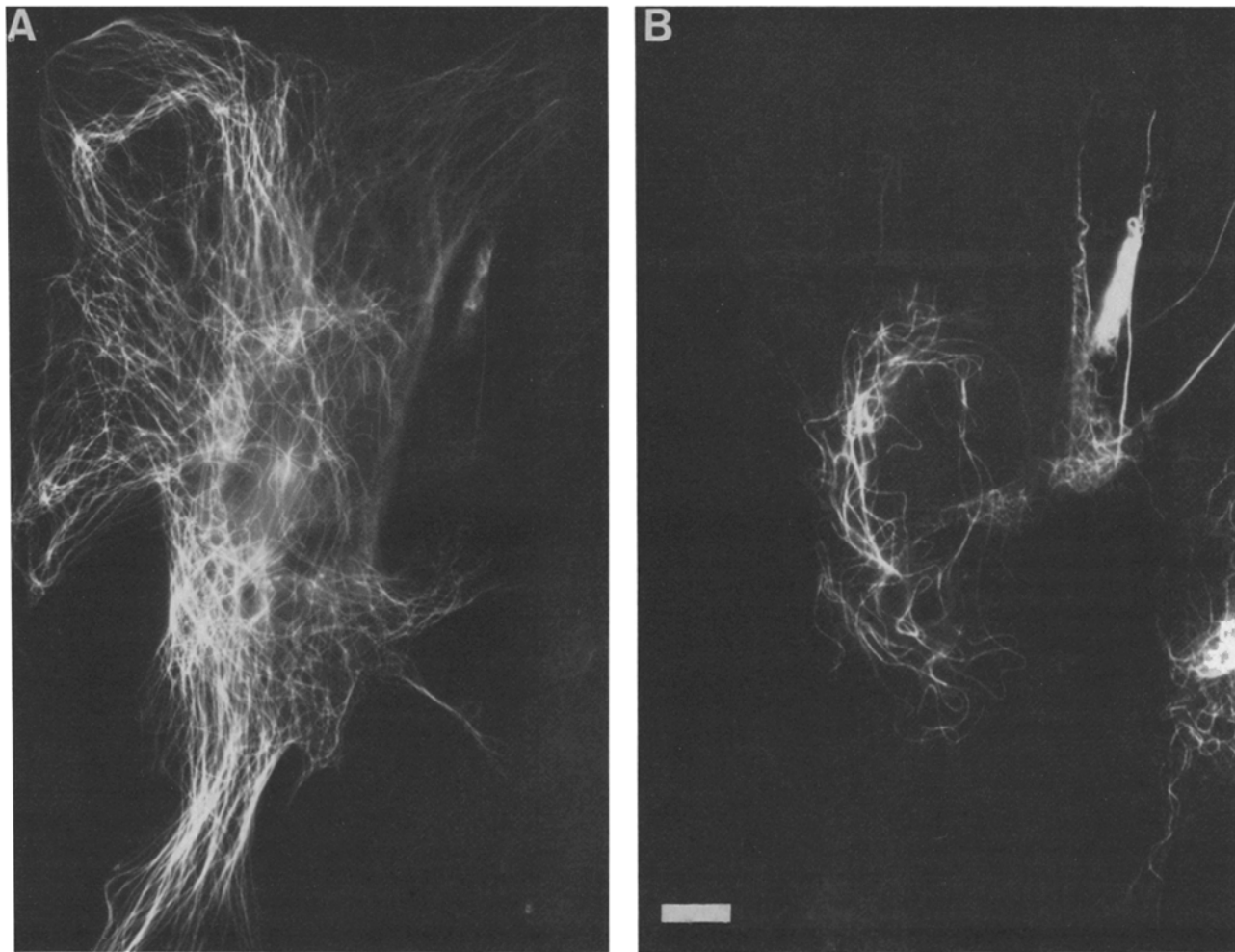
As a control, we also tested whether there are any microtubules in the above cells that were not masked by the anti-Glu primary and subsequent antibody layers and therefore could be stained by antitubulin. As expected based on previous double-label immunofluorescence experiments, the majority of the cells' microtubules were stained with antitubulin (see Table II), and therefore had not been masked with the initially applied antibody to posttranslationally modified tubulin.

#### ***Relationship between Stable and Posttranslationally Modified Microtubules***

***Stable Microtubules Are Posttranslationally Modified in SKNSH Cells.*** With the relationship between the acetylated and Glu microtubules in SKNSH cells clear, we next began to probe the relationship between the stable microtubules and the acetylated and Glu microtubules in SKNSH cells. Our first question was whether there were any stable microtubules in these cells that were posttranslationally modified. This was done by microinjecting SKNSH cells with biotin-labeled tubulin, followed by an incubation period of 1 h during which the biotin-labeled tubulin was incorporated into the cells' dynamic microtubules (Schulze and

Kirschner, 1986, 1987). Using antibody-blocking protocol C detailed in Table I, we permeabilized and fixed the cells, then incubated with antibiotin antibody followed by fluorescein-labeled secondary, and tertiary antibodies. This procedure allowed the visualization of all the microtubules that grew during the period of incubation, and therefore incorporated biotin-tubulin (Fig. 4 A). These microtubules were further reacted with unlabeled quaternary and quinary antibodies, masking them from further reaction with other primary antibodies as in previous experiments. This left only the stable (nonexchanged) microtubules unmasked and therefore available for staining with the second primary antibody. The cells were then reacted with anti-acetylated-tubulin antibody followed by staining with the appropriate Texas Red-labeled secondary antibodies. Fig. 4 B is an immunofluorescence image of acetylated tubulin in an injected cell, revealing only the stable microtubules that were also acetylated. When 76 such cells were examined, we found acetylated microtubules in all cells and in approximately the same numbers ( $\sim$ 10–20% of the population) as expected for stable microtubules regardless of their posttranslational modifications (see Table II). It was not possible to do the same experiment of blocking and staining with antibody to the Glu tubulin because both that antibody and the antibiotin antibody were made in rabbits. However since the previous series of experiments showed that Glu and acetylated modifications were coextensive, we can assume that stable microtubules are also Glu.

***There Are No Unmodified Stable Microtubules in SKNSH Cells.*** Clearly there are many stable microtubules that are also posttranslationally modified, however, we do not know whether there are any stable microtubules that are not posttranslationally modified. To answer this question, we performed a further variation of the above experiment (antibody-blocking protocol D detailed in Table I). SKNSH cells were microinjected with biotin-labeled tubulin, incubated, fixed, and permeabilized as before. They were then



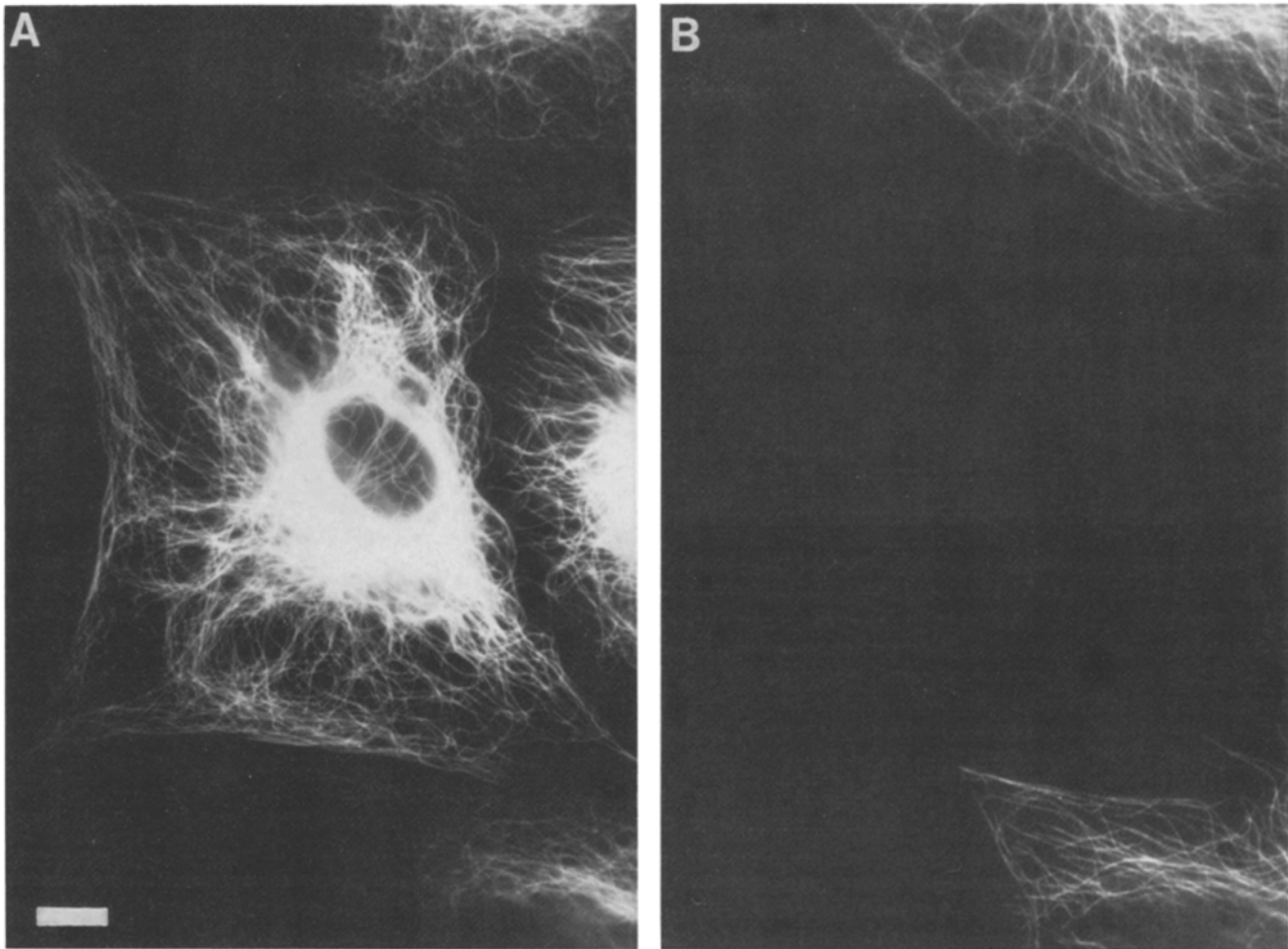
**Figure 4.** Immunofluorescence micrographs of a SKNSH cell microinjected with biotin-tubulin, and incubated 1 h before preparation for antibody-blocking protocol C. (A) Antibiotin immunofluorescence revealing the masked, biotin-labeled dynamic (exchanged) microtubules. (B) Anti-acetylated-tubulin immunofluorescence revealing the acetylated, stable (nonexchanged) microtubules. Bar, 10  $\mu$ m.

sequentially reacted with both antibiotin and anti-Glu primary antibodies followed by the appropriate series of blocking antibodies (see Materials and Methods) to mask both the dynamic (biotin-tubulin containing) and Glu microtubules from further reaction with any primary antibodies. In such injected double-blocked cells, antitubulin immunofluorescence should reveal any nondynamic, non-Glu microtubules. Fig. 5 shows the antibiotin/anti-Glu compound immunofluorescence of an injected SKNSH cell revealing both the dynamic and the Glu microtubules. The microtubules have the typical distribution of the total microtubule population in these cells. Fig. 5 B shows the antitubulin immunofluorescence of the same cell indicating that there are no stable microtubules that can now be reacted with antitubulin. Of 15 such cells examined, none showed evidence of stable microtubules that were not posttranslationally modified (Table II).

**There Are No Dynamic Posttranslationally Modified Microtubules in SKNSH Cells.** We know that all stable microtubules are posttranslationally modified (thus the stable microtubules are at least a subset of the posttranslationally modified microtubules), but we do not know whether all posttranslationally modified microtubules are stable. One

can address this question by asking whether any growing microtubules are posttranslationally modified. Previous experiments (Schulze and Kirschner, 1986) have shown that stable microtubules do not in general incorporate even small amounts of biotin tubulin at their tips. To ask whether growing microtubules are posttranslationally modified, cells were injected with biotin-tubulin, incubated for a short time (<5 min) and then stained for both biotin-tubulin, and acetylated tubulin. We then asked whether any of the growing microtubules were acetylated. Because of the high density of biotin-labeled microtubule segments and acetylated microtubule ends, we used two different counting methods to check for growing acetylated microtubules. By the first method, we checked 77 unambiguous biotin-labeled microtubule segments in four injected cells and found that none were at the ends of acetylated microtubules (see Fig. 6, *arrows*). By the second method, we checked 39 unambiguous acetylated microtubule ends in six injected cells, and found none had biotin-tubulin segments at their ends (see Fig. 6, *stars*). This indicated that in SKNSH cells, growing microtubules are not acetylated, and acetylated microtubules do not grow. However, we emphasize that in contrast to the previous experi-





**Figure 5.** Immunofluorescence micrographs of a biotin-tubulin-injected SKNSH cell incubated 1 h before preparation using the antibiotic, anti-Glu double block (protocol D). (A) Antibiotin, anti-Glu compound immunofluorescence revealing both the dynamic (exchanged) and Glu microtubules. (B) Antitubulin immunofluorescence revealing the injected cells' lack of stable (nonexchanged) non-Glu (tyrosinated) microtubules. Note the cell to the right of the central cell is also injected. Bar, 10  $\mu\text{m}$ .

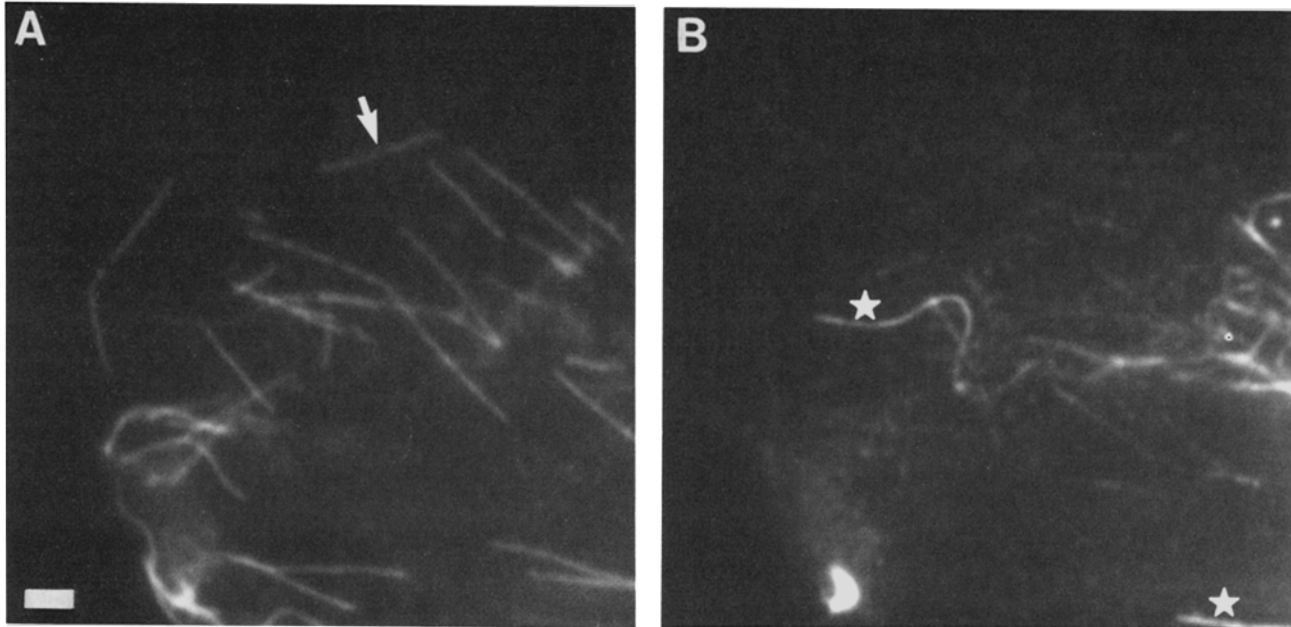
ments identification of microtubule ends is possible for only a small fraction of microtubules in a single cell. Therefore with this caveat we can say that because all stable microtubules are posttranslationally modified, and posttranslationally modified microtubules do not grow, the two sets must be the same set. We can conclude, then, that in SKNSH cells, stable, acetylated, and Glu microtubules are equivalent subsets of the cells' total microtubule population.

#### ***Variations of Posttranslational Modification and Microtubule Stability in Other Cells***

Cell lines appear to be quite variable in the extent to which they incorporate posttranslational modifications in their microtubules (Piperno, 1987). Ptk cells, for example, do not acetylate their microtubules detectably even after taxol treatment (Piperno et al., 1987) while chick embryo fibroblasts appear to acetylate them extensively (Asai, D. J., personal communication). We wished to know the relationship between posttranslational modifications and microtubule stability in these two cell types. Ptk cells incorporate biotin-tubulin at similar rates to SKNSH cells (data not shown). It was unknown whether they have a class of stable microtu-

bules, despite their lack of acetylation. Fig. 7 A shows the antibiotic immunofluorescence of a Ptk1 cell that had been injected with biotin-tubulin and incubated for 1 h before fixation, revealing dynamic microtubules. Fig. 7 B shows the antitubulin immunofluorescence in the same cell of those microtubules that had not exchanged (see protocol E in Table I). Clearly, there is a subset of stable microtubule in this cell.

In virtually all chick embryo fibroblast cells the majority of microtubules are detyrosinated (Fig. 8 A) with many cells having a majority of their microtubules highly acetylated (Fig. 8 B). Using biotin-tubulin injection we could ask if there was a corresponding large number of stable microtubules. Fig. 9 A shows the dynamic microtubules visualized with antibiotic immunofluorescence, while Fig. 9 B shows the stable microtubules in the same cell (see also protocol E in Table I). To our surprise, these cells appeared to have very few stable microtubules compared with BSC1, Ptk1, and SKNSH cells. Of 24 cells examined, 14 had no microtubules stable to exchange for 1 h, 7 had fewer than 6 per cell, and 3 had <10% unexchanged microtubules. We therefore conclude that it is possible to accumulate posttranslational modifications without corresponding stabilization, that acet-



**Figure 6.** Immunofluorescence of a small part of an SKNSH cell which was microinjected with biotin-tubulin and incubated for <5 min. (A) Antibiotin immunofluorescence revealing newly growing microtubule ends. (B) Anti-acetylated-tubulin immunofluorescence revealing the acetylated microtubules. Arrow points to an unambiguous biotin-tubulin segment which is clearly not coextensive with an acetylated microtubule. Stars mark two acetylated microtubules which are not biotin labeled. (Note these acetylated microtubules are faintly visible in the anti-biotin micrograph because of bleed through of the Texas Red into the fluorescein channel. Bar, 2  $\mu$ m.

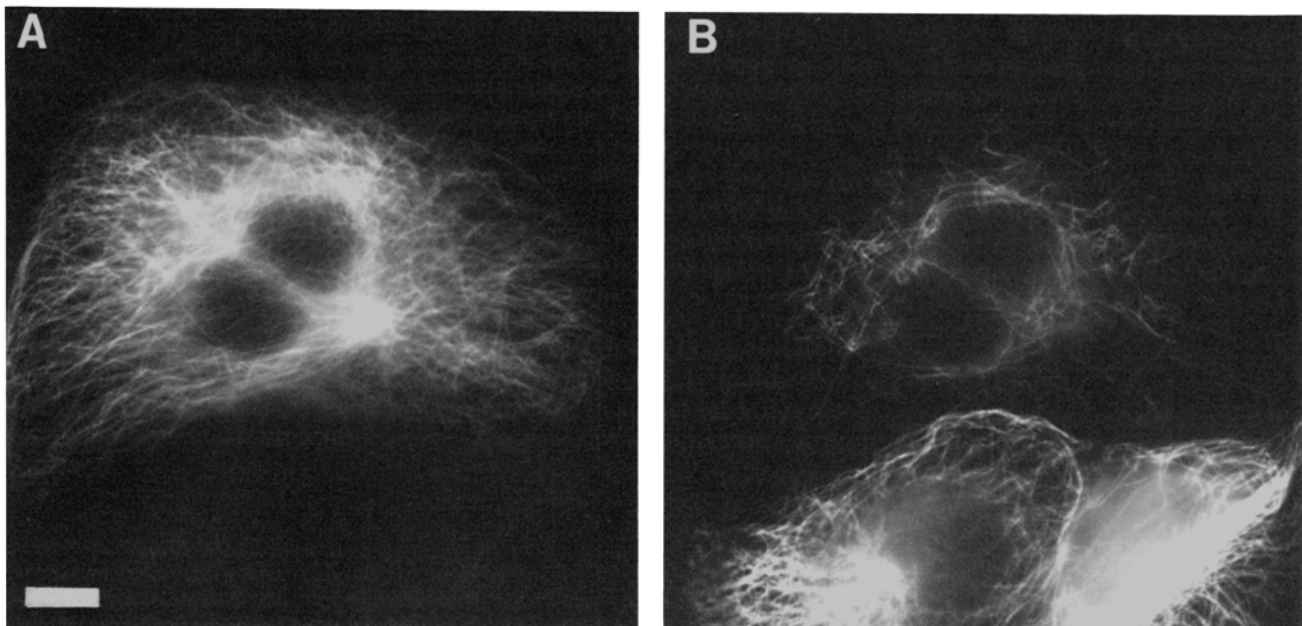
ylation is not necessary for microtubule stability in Ptk1 cells, and that detyrosination is not sufficient to cause microtubule stability in chick embryo fibroblast cells.

### Discussion

Microtubules in cells have been shown to exist in different classes based on their rates of turnover (Schulze and Kirsch-

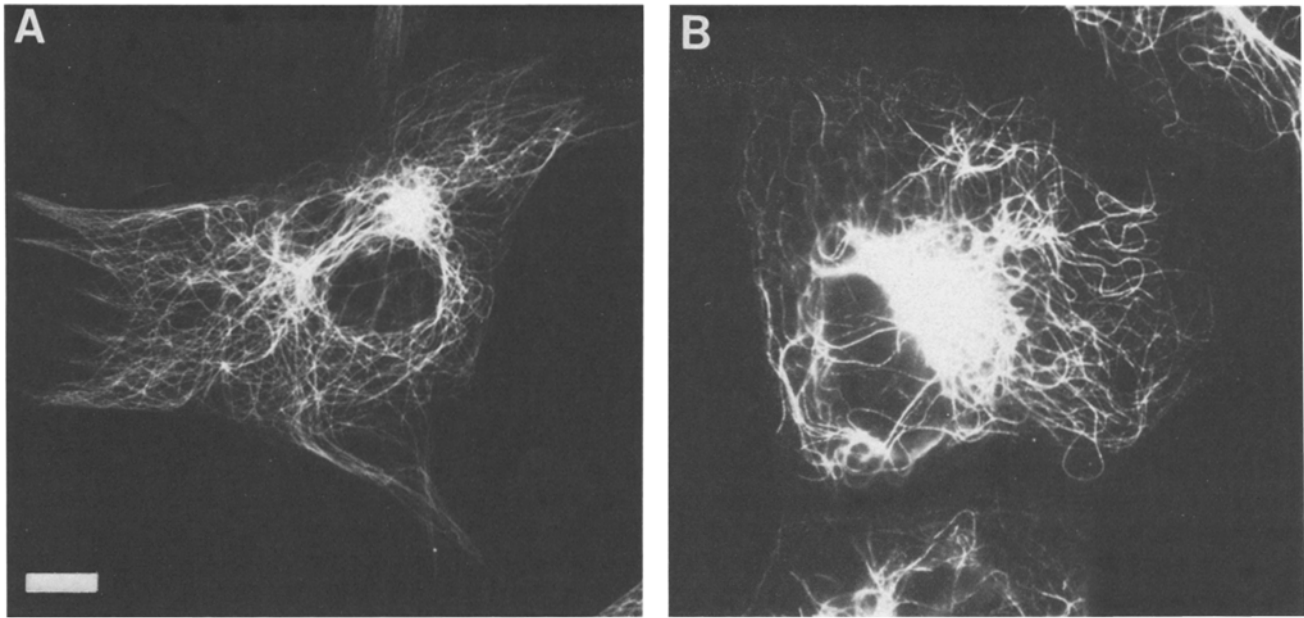
ner, 1987) and based also on their posttranslational modification (Thompson et al., 1984; Gundersen et al., 1984; Piperno, 1987). We have been interested in the relationship among these classes and the possible role of microtubule classes in morphogenesis.

We used human retinoblastoma cells for our initial analysis because they consistently contained a fairly uniform population of stable, Glu, and acetylated microtubules. Our



**Figure 7.** Immunofluorescence micrograph of a Ptk1 cell injected with biotin-tubulin and incubated for 1 h before preparation using the antibody-blocking technique (protocol E). (A) Antibiotin staining showing the exchanged (dynamic) microtubules. (B) Antitubulin staining revealing an extensive network of unexchanged (stable) microtubules. Bar, 10  $\mu$ m.



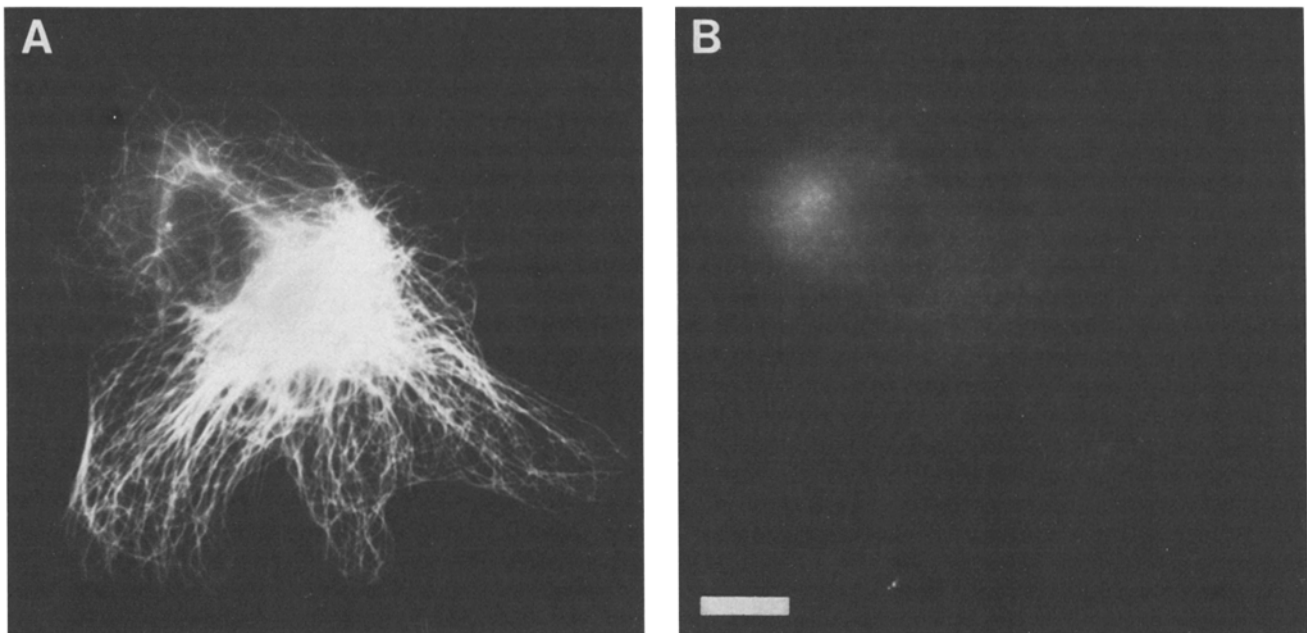


**Figure 8.** Immunofluorescence micrographs of chick embryo fibroblast cell. (A) Anti-Glu immunofluorescence revealing the Glu (detyrosinated) microtubules in this cell. (B) Anti-acetylated-tubulin immunofluorescence revealing the acetylated microtubules in a different chicken embryo fibroblast cell. Bar, 10  $\mu\text{m}$ .

first experiments were designed to probe the relationship between the acetylated and Glu microtubules at the level of single microtubules. We employed a variation of the antibody-blocking protocol originally used to distinguish different classes of microtubule stability. In this variation, microtubules were first masked with an antibody to one form of modified tubulin (acetylated tubulin), and then stained with an antibody to the second (Glu tubulin). The converse of this experiment was also performed: masking Glu microtubules,

and staining acetylated microtubules. In neither case did the second antibody stain any microtubules at all. Superficially the first experiment would indicate that there was no Glu tubulin in any microtubule that did not contain acetylated tubulin. It is more accurate to say, however, that there was no detectable Glu tubulin in any microtubule that did not possess enough acetylated tubulin to allow it to be masked in the blocking protocol.

The threshold for detection and for masking is difficult to



**Figure 9.** Immunofluorescence micrograph of a chicken embryo fibroblast cell injected with biotin-tubulin and incubated for 1.5 h before preparation using the antibody-blocking technique (protocol E). (A) Antibiotin staining showing the exchanged (dynamic) microtubules. (B) Antitubulin staining revealing the cells' lack of stable microtubules. Bar, 10  $\mu\text{m}$ .

determine. Our only data on the molar ratios of modified to unmodified tubulin necessary for immunofluorescence visualization, and for antibody blocking, come from biotin-tubulin injection experiments. In these experiments we could control the ratios of unmodified and modified tubulin (but not their efficiencies of incorporation into microtubules) and have found empirically that a molar ratio of one biotin-labeled tubulin subunit to 40 unlabeled tubulin subunits (including both injected tubulin and that present in the cell) is the minimum level necessary to detect a biotin-labeled microtubule by immunofluorescence. Similarly a molar ratio of one biotin-labeled to ten unlabeled subunits is the minimum necessary to mask a biotin-labeled microtubule from further reaction with other antitubulin antibodies. Because the level of detection is quite low the complementary blocking experiments with anti-Glu and acetylated antibodies would seem to indicate that microtubules in SKNSH cells exist in only one of two states with respect to the molar ratio of modified to unmodified tubulin. In one state there is a minimum of ~10% modification and in the other there is <3% modification. These estimates are, of course, imprecise, and the signal may be influenced by the relative geometry and affinities of the two epitopes. The results suggest, however, that microtubules in these cells are divided into two classes. One class contains very low levels of posttranslationally modified tubulin. The other class contains microtubules that are significantly modified by both acetylation and detyrosination. There are two interesting conclusions which can be drawn about this second class. First, any microtubule which is modified to the level of detection is also modified to a level high enough to block. The absence of microtubules that are detectable but cannot block suggests that there is not a continuous distribution of modified microtubules in these cells. Therefore, there is a class of relatively unmodified microtubules and a class of relatively highly modified microtubules, but no class with intermediate levels of modifications. The second interesting point is that all microtubules that are detyrosinated above the level of detection and blocking are also acetylated above the level of detection and blocking and vice versa.

It is simplest to imagine that a common mechanism might specify which microtubules are modified and that this mechanism could be related to differential microtubule stability. We had previously discovered a subset of microtubules that was stable to exchange with free tubulin for 1 or 2 h and that had a distribution and morphology very similar to the aforementioned modified microtubules. The half-life for these "stable" microtubules was ~1 h as opposed to 5–10 min for the dynamic microtubules. These stable microtubules were therefore, on average, older than the dynamic microtubules. Since posttranslational modification of tubulin is a time-dependent process that occurs preferentially on the microtubule polymer (Gundersen et al., 1987), stable microtubules, which in general are older should be more highly modified.

To test this prediction we needed to evaluate each microtubule in the cell both in terms of its stability and its state of modification. After microinjection with biotin-tubulin, incubation for 1–2 h and masking with antibiotin-tubulin antibody and a series of secondary antibodies, only the stable, nonexchanged, and therefore, older microtubules were available for staining with another antibody to tubulin. We stained with antibody to acetylated tubulin, and found that there were indeed older modified microtubules. This experiment

did not rule out the existence of older unmodified microtubules. Their lack of existence was an important test of the correlation between stability and modification. To test for unmodified stable microtubules, cells were injected with biotin-tubulin, incubated, and blocked with both antibiotin and anti-Glu antibodies. When the existence of both nonexchanged, unmodified microtubules was evaluated by staining with antitubulin, no such unmodified stable microtubules could be found. In support of the validity of this complex experiment one can see antitubulin staining of undiminished intensity in the surrounding uninjected cells.

Although these last two experiments demonstrate that all stable microtubules are post-translationally modified, the possibility still exists that there are dynamic microtubules that are also modified. To identify newly assembled microtubules, cells were injected with a short pulse of biotin-tubulin which produced short segments at the ends of growing microtubules. By double-label immunofluorescence against biotin, and acetylated tubulin, we found that growing microtubules are not acetylated, and furthermore, that acetylated microtubules do not appear to grow. Unlike the previous experiments reported here, only a small fraction of microtubules could be evaluated near the periphery of the cell. However, the total absence of any dynamic modified microtubules suggests that this distinction is probably true for all microtubules in this cell type.

A simple model for the relationship between microtubule subsets in human retinoblastoma cells, based on the above results is that microtubules exist in two kinetically distinct populations. Dynamic microtubules rapidly grow using free unmodified tubulin, and frequently shrink, never existing long enough to be significantly (detectably) modified. Stable microtubules, by virtue of being older, simultaneously accumulate significant concentrations of both forms of post-translationally modified tubulin thereby accounting for the equivalence of all three microtubule subsets. The model would predict that there would not be a strict absence of modified subunits in the dynamic population and in fact immunolocalization of tyr and Glu tubulin in the same microtubules has been observed (Geuens et al., 1986). This model and the above results predict that the period of time needed to modify a microtubule for both detection, and masking be very nearly the same for both posttranslational modifications (the subsets would not overlap otherwise), and also greater than the half-life of the dynamic microtubules (or there would be many modified and dynamic microtubules present). Based on a preliminary experiment where we noted the first appearance of biotin label and detyrosination in the same microtubule, we estimate the period of time necessary for a microtubule to reach the detection threshold as ~15–20 min. This agrees with recent data of Gundersen et al. (1987) who found a half-time of ~25 min for African Green Monkey kidney cells. This contrasts with the half-life of a dynamic microtubule estimated to be between 1.5 and 5 min by photobleaching, and 5 and 10 min from our previous microinjection experiments.

These correlations really do not answer the questions of whether posttranslational modifications are the cause, consequence or are merely incidental to the existence of a stable nongrowing class of microtubules in the cell. In Ptk1 cells where there appear to be few or no acetylated microtubules (due possibly to the absence of an acetylase or the presence of an exceptionally active deacetylase or some other reason)

a stable class of microtubules exists. Acetylation therefore cannot be the cause of differential stabilization in these cells. In chick embryo fibroblasts there is extensive deetyrosination and variable acetylation. Yet these cells turn over their microtubules with roughly the same kinetics as other cells and in fact have an exceptionally small class of stable microtubules. We do not know whether there are other factors in these chick cells that increase turnover despite the extensive modification, but the simplest explanation for both of these examples is that deetyrosination and most likely acetylation do not affect stability. This conclusion is consistent with the few *in vitro* experiments that have failed to find significant alterations of microtubule assembly due to acetylation and deetyrosination (Maruta et al., 1986; Raybin and Flavin, 1977). Whether stable microtubules are a subset of modified microtubules or vice versa or whether they are completely congruent as in SKNSH cells must then depend on the relative rates of modification and microtubule turnover. For example, if modification were rapid relative to the microtubule half-life, the dynamic microtubules as well as the stable microtubules would accumulate posttranslational modifications. If the rate of modification were very slow only the most stable microtubules would accumulate the modification. It is important to point out that at no time did we find modified tubulin preferentially in the dynamic population, which would have been inconsistent with this view.

What purpose would there be to first generate a stable population of microtubules and then modify them? Selective stabilization of microtubules has been discussed as a general model for morphogenesis (Kirschner and Mitchison, 1986; Kirschner and Schulze, 1986). In this model, a cell in the absence of an outside morphogenetic signal, randomly polymerizes microtubules in all directions with the rapid turnover predicted by the property of dynamic instability *in vitro* (Mitchison and Kirschner, 1984) as well as the behavior observed in living cells (Saxton et al., 1984; Schulze and Kirschner, 1986). A signal received at the cell surface might be transduced into the local stabilization of microtubules at the site of signal reception. In this paper we have provided further evidence that stable nongrowing microtubules exist in cells in close proximity to dynamic ones although we have no idea how these microtubules are stabilized. However, for their stability to have some function it must be converted into a form of spatial differentiation. One means by which microtubule stability could be transduced into a structural differentiation of the microtubule would be via a time-dependent posttranslational modification (Kirschner and Mitchison, 1986). This could lead to further microtubule differentiation on the basis of further modifications such as phosphorylation or MAP binding that could possibly, but not necessarily, lead to further stabilization. The structurally differentiated microtubules might thereby become functionally differentiated leading to changes in their interaction with cellular components. The experiments reported here also indicate that the extent of structural differentiation can be controlled by both changes in microtubule stability and changes in the rates of posttranslational modification. In this view stabilization is the primary determinant of the location and extent of posttranslational modification. The ability of the cell to modulate the rates of microtubule modification, and microtubule stability, gives cells the ability to create many different combinations of the specific microtubule subsets. In the three cell types studied, we have found three different

combinations of microtubule subsets. The function of the subsets remains unknown, as is the mechanism of stabilization but it seems likely that both stability and modification are intimately involved in cell morphogenesis.

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