

# Enhanced Stability of Microtubules Enriched in Detyrosinated Tubulin Is Not a Direct Function of Detyrosination Level

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**Abstract.** Interphase cultured monkey kidney (TC-7) cells contain distinct subsets of cellular microtubules (MTs) enriched in posttranslationally detyrosinated (Glu) or tyrosinated (Tyr)  $\alpha$  tubulin (Gundersen, G. G., M. H. Kalnoski, and J. C. Bulinski. 1984. *Cell*. 38:779-789). To determine the relative stability of these subsets of MTs, we subjected TC-7 cells to treatments that slowly depolymerized MTs. We found Glu MTs to be more resistant than Tyr MTs to depolymerization by nocodazole in living cells, and to depolymerization by dilution in detergent-permeabilized cell models. However, in cold-treated cells, Glu and

Tyr MTs did not differ significantly in their stability. Digestion of permeabilized cell models with pancreatic carboxypeptidase A, to generate Glu MTs from endogenous Tyr MTs, did not significantly alter the resistance of the endogenous Tyr MTs toward dilution-induced depolymerization. Furthermore, in human fibroblasts that contained no distinct Glu MTs, we observed a population of nocodazole-resistant MTs. These data suggest that Glu MTs possess enhanced stability against end-mediated depolymerization, yet detyrosination alone appears to be insufficient to confer this enhanced stability.

A unique posttranslational modification of  $\alpha$  tubulin has been described in which a tyrosine residue is reversibly added to the COOH terminus (Barra et al., 1973, 1974). Two enzymes are involved in these modifications: a tubulin tyrosine ligase, which adds a tyrosine residue to the COOH terminus (Raybin and Flavin, 1977a; Flavin and Murofushi, 1984), and a tubulin carboxypeptidase, which removes the COOH-terminal tyrosine (Argarana et al., 1978, 1980). No dramatic differences have been detected between the in vitro behavior of tyrosinated (Tyr)<sup>1</sup> and detyrosinated (Glu) tubulin (Raybin and Flavin, 1977b; Kumar and Flavin, 1982; Arce et al., 1978). Nonetheless, changes in the relative levels of Tyr and Glu tubulin or in the activity of the enzymes carrying out the modifications occur during differentiation (Barra et al., 1973, 1980; Deanin et al., 1977; Nath and Flavin, 1979; Rodriguez and Borisy, 1978, 1979), during the cell cycle (Forest and Klevecz, 1978), and in at least one disease condition (Nath et al., 1982). To date, the role of posttranslational tyrosination in microtubule (MT) function has not been determined.

Recently, we localized Tyr and Glu tubulin in cultured cells using antibodies specific for each form of tubulin (Gundersen et al., 1984). Curiously, these two species were found to be predominantly segregated into distinct populations of

microtubules; that is, most MTs contained primarily Tyr tubulin (Tyr MTs), while a few MTs contained primarily Glu tubulin (Glu MTs) (Gundersen et al., 1984; Geuens et al., 1986). Functional differences between Tyr and Glu MTs, though not established, are suggested by the observation that Tyr tubulin predominates in the dynamic MTs of proliferating cells, while Glu tubulin predominates in the stable assemblies of MTs present in differentiated cells, e.g., flagellar and ciliary axonemes, neurites, and marginal bands (Gundersen and Bulinski, 1986a).

An important question that arises is whether Glu and Tyr MTs exhibit different properties in vivo. Differences in the behaviors of Glu and Tyr MTs would, in turn, imply functional differences between the different types of MTs. Tyrosination/detyrosination is a feasible mechanism for creating functionally distinct subsets of MTs, since in a previous study we demonstrated that MTs with biochemically different tubulin subunits were created in vivo by postpolymerization detyrosination (Gundersen et al., 1987a). In interphase cells, most MTs are dynamic, turning over with a half-life of 5-10 min; in contrast, some MTs appear to exhibit a very slow rate of turnover, with a half-life of 1 h or more (Schulze and Kirschner, 1986, 1987). The existence of a small population of stable MTs in interphase cells has also been suggested by studies using MT-depolymerizing agents in cultured cells (Thompson et al., 1984; Piperno et al., 1987), and in monocytes (Cassimeris et al., 1986). Currently, both the mechanism for the stabilization of a small subset of interphase MTs and the functional significance of these stabilized MTs are unknown.

1. *Abbreviations used in this paper:* CPA, pancreatic carboxypeptidase A; Glu, detyrosinated; MAP, microtubule-associated protein; MT, microtubule; PEM buffer, 85 mM Pipes, pH 6.94, 10 mM EGTA, and 1 mM MgCl<sub>2</sub>; Tyr, tyrosinated.

In this paper we have determined the relative stability of Glu and Tyr MTs in African green monkey kidney (TC-7) cells to three different depolymerization treatments: nocodazole and cold treatments of intact cells, and dilution of detergent-extracted cytoskeletons. In addition, we examined cells and cytoskeletons with dramatically different levels of Glu tubulin in their MTs in order to determine the effect of detyrosination on microtubule stability.

## Materials and Methods

### Cell Culture and Treatments

TC-7 cells, an epithelial-like cell line derived from the CV-1 line of African green monkey kidney cells, were grown in DMEM supplemented with 10% FCS (growth medium), as previously described (Gundersen et al., 1984). Human foreskin fibroblast cells (strain 356) derived by R. De Mars (University of Wisconsin, Madison, WI), were cultured in F-10 medium supplemented with 10% FCS. Cells were seeded onto glass coverslips in 100-mm petri dishes and allowed to grow for 2–3 d before experiments.

Nocodazole treatments were performed by adding the drug to growth medium to achieve a final concentration of 0.2 or 0.6  $\mu\text{g/ml}$ , using a nocodazole stock prepared in DMSO (the final DMSO concentration did not exceed 0.1%, and this concentration of DMSO had no effect on the stability of Glu or Tyr MTs). Cold treatments were performed at 8°C, using a circulating waterbath (Brinkmann Instruments Co., Westbury, NY); 8°C was chosen since depolymerization at 0°C was too rapid ( $t_{1/2} \sim 1$  min) to monitor conveniently. Human 356 cells were treated with 0.3  $\mu\text{g/ml}$  of nocodazole in growth medium for 30, 60, or 90 min, then they were rinsed in nocodazole-containing buffer, and immediately fixed in cold methanol.

### Extraction and Fixation

TC-7 cells from all treatments (except untreated cells in the dilution experiments, as noted below) were extracted to remove monomeric tubulin, whose bright diffuse staining would otherwise obscure MTs. All steps in the extraction protocol were performed at 25°C unless noted otherwise. Coverslips were rinsed twice in PEM buffer (85 mM Pipes, pH 6.94, 10 mM EGTA, and 1 mM  $\text{MgCl}_2$ ), and then extracted for 1 min with 8 ml of PEM (per 100-mm dish) containing 0.1 (for nocodazole-treated cells) or 0.5% (for cold-treated cells) Triton X-100. The extraction of nocodazole-treated cells was performed in buffer supplemented with nocodazole at the same concentration as the treatment to prevent repolymerization of microtubules during extraction. Similarly, cold-treated cells were extracted with buffer maintained at 8°C. After extraction, cells were rinsed briefly in PEM and fixed in methanol (–20°C, 5 min).

To examine the stability of Glu and Tyr MTs to dilution-induced depolymerization, cells were extracted with 0.1% Triton X-100 at 25°C as above, rinsed twice in PEM, and then incubated in PEM at 37°C for various intervals before methanol fixation. For some experiments, extracted cells were rinsed to remove the Triton X-100 and then treated with 50  $\mu\text{g/ml}$  of carboxypeptidase A (CPA; Cooper Biomedical, Inc., Malvern, PA) for 5 min at 37°C in PEM buffer. The CPA-treated cytoskeletons were rinsed twice with PEM and further incubated in PEM buffer at 37°C before methanol fixation.

### Indirect Immunofluorescence

Double-indirect immunofluorescence was performed as described (Gundersen et al., 1987a) except that the Glu and Tyr antibodies were used at 1:100 dilutions. The Glu antibody was a rabbit polyclonal peptide antibody specific for Glu tubulin (Gundersen et al., 1984) and the Tyr antibody was a rat monoclonal antibody (YL 1/2) specific for Tyr tubulin (Kilmartin et al., 1982; Wehland et al., 1983). YL 1/2 was generously provided by Dr. J. V. Kilmartin (Medical Research Council, Cambridge, England). Total cellular tubulin was visualized by staining with a mouse monoclonal antibody specific for  $\beta$  tubulin (designated 3F3) at 1:100 dilution of ascites fluid (Moyer et al., 1986). 3F3 was kindly provided by Dr. J. Lessard (University of Cincinnati, Cincinnati, OH). Secondary antibodies were fluorescein-conjugated goat anti-rat IgG, rhodamine-conjugated goat anti-rabbit IgG, and fluorescein-conjugated goat anti-mouse IgG (obtained from Cooper Biomedical, Inc.) and were used at 1:20 dilutions. No difference was observed if the fluorochromes on each secondary antibody were switched.

Incubations with Glu and Tyr antibodies or Glu and 3F3 antibodies were performed simultaneously, as were subsequent incubations with the secondary antibodies.

### Quantification of Microtubules

Cells stained as above were examined with a Nikon epifluorescence microscope equipped with the appropriate filters for fluorescein and rhodamine fluorescence. Images were recorded on 35 mm Kodak Tri-X film using a 40 $\times$  (1.0 NA) or a 63 $\times$  (1.4 NA) Zeiss planapochromat lens and exposure times of 4–16 s. The film was developed to ASA 400 with HC-110 developer and negatives were printed at a magnification of 870 (about twice the magnification of the figures in Results). The number of Glu and Tyr MTs per cell was determined by tracing individual Glu- (or Tyr-) stained MTs visible on photographic prints onto acetate sheets and then counting the traces. All coverslips were stained with the same preparations of Glu and Tyr antibodies, at the same concentration of antiserum. Thus, consistent criteria were used to identify and count Glu and Tyr MTs in each cell and preparation of cells examined.

For each time point, we determined the number of Glu and Tyr MTs in 30–44 randomly chosen cells. The mean from two or three separate experiments was then used to prepare the curves in Results. Certain cells were excluded from analysis because of either their unusual MT number (giant, multinucleated cells), or their known lack of Glu MTs (early G<sub>1</sub> cells still connected by midbodies or early prophase cells with split centrosomes and condensing chromatin) (Gundersen and Bulinski, 1986b).

## Results

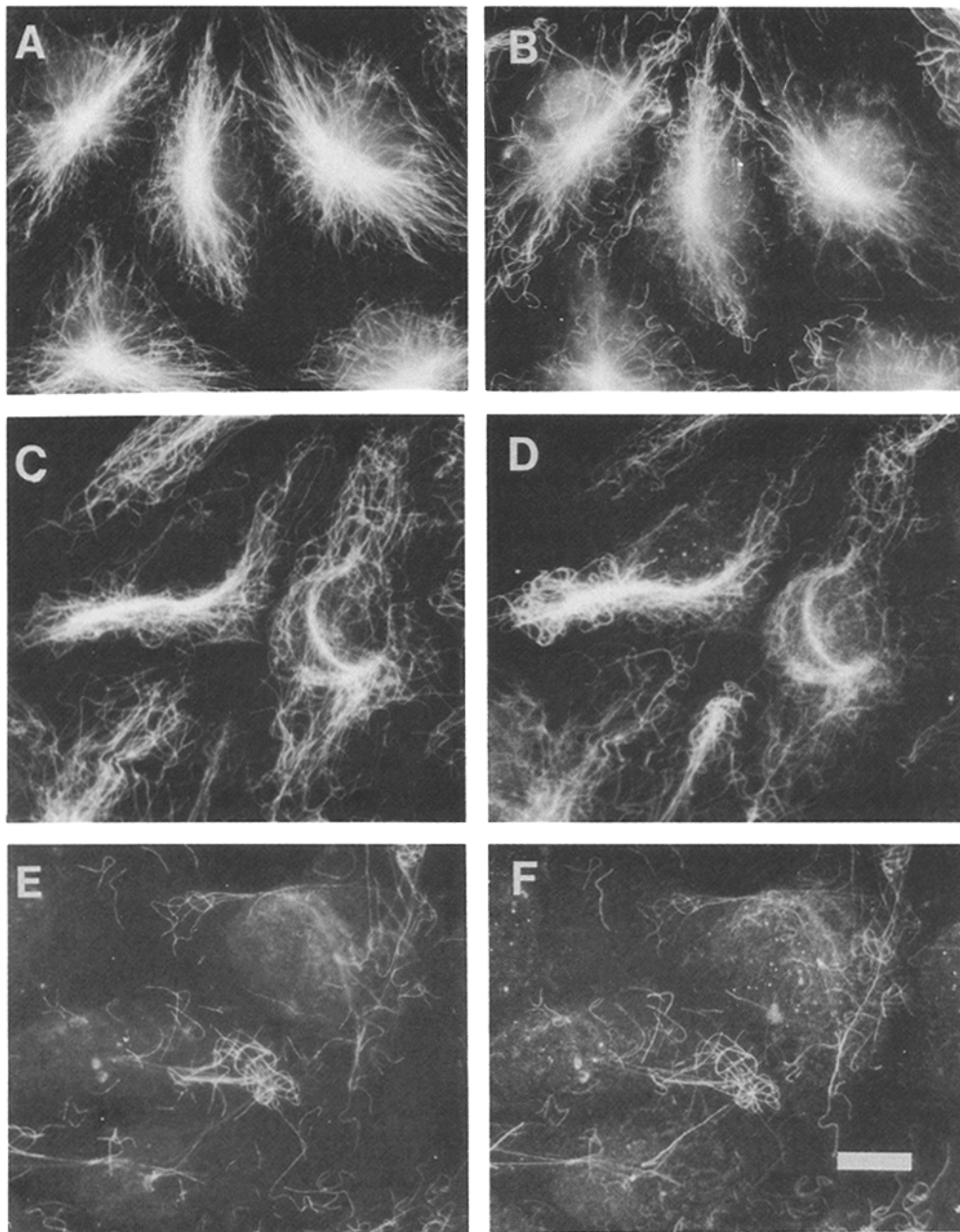
MTs containing Glu tubulin are generated by the post-polymerization detyrosination of Tyr MTs (Gundersen et al., 1987a). Thus, long-lived MTs would be expected to contain a relatively high content of Glu tubulin when compared with short-lived MTs. This prediction is borne out by the elevated levels of Glu tubulin in stable assemblies of MTs in both proliferating and differentiated cells (Gundersen and Bulinski, 1986a). An important question is whether the detyrosination of MTs contributes to their stability or whether it is merely a consequence of the stability induced by other factors. We sought to test this question by examining the stability of Glu and Tyr MTs *in vivo* to a variety of treatments that depolymerize MTs.

We have used the interphase array of MTs in TC-7 cells, an epithelial-like cell line, as the source of MTs, since these cells contain well-defined, co-existing populations of Glu and Tyr MTs in their cytoplasm (Gundersen et al., 1984, 1987a). Although there is probably a continuum of different levels of Glu and Tyr tubulin in cellular MTs (Geuens et al., 1986), by double immunofluorescence some MTs in TC-7 cells stain only with a Glu-specific antibody (these are termed Glu MTs), while others stain only with a Tyr-specific antibody (these are termed Tyr MTs; Gundersen et al., 1984). In both cases, the lack of immunofluorescent staining presumably indicates that the level of that species is too low to be detected. A third class of MTs are those that exhibit immunofluorescent staining with both antibodies (Glu-Tyr MTs). Ideally, it would be interesting to determine the stability of each of these three classes; however, because of the difficulty of accurately counting MTs in cells, we have restricted our study to a comparison between MTs that stain with the Tyr antibody (Tyr and Glu-Tyr MTs) and those that stain with the Glu antibody (Glu and Glu-Tyr MTs). For convenience, in the remainder of the paper we will refer to these two categories as Tyr and Glu MTs, respectively.

### Stability of Tyr and Glu MTs to Depolymerization by Nocodazole Treatment

Tyr and Glu MTs in TC-7 cells can be completely depolymerized by prolonged treatment with moderate concentrations of nocodazole (Gundersen et al., 1987a). However, if lower concentrations of nocodazole are used, MTs depolymerize slowly, allowing the number of Tyr and Glu MTs to be determined at various times of treatment. Fig. 1, *A* and *B*, show the distributions of Tyr and Glu MTs, respectively, in a typical field of untreated cells. After a 15-min treatment with 0.2  $\mu\text{g/ml}$  nocodazole, both types of MTs were clearly reduced in number (Fig. 1, *C* and *D*). With longer treatments, further MT breakdown occurred, so that by 90 min only a small number of MTs remained in each cell (Fig. 1, *E* and *F*). Determinations of the number of Tyr and Glu MTs in 10 repre-

sentative cells at each of three time points are shown in Table I. The individual MT counts for both Tyr and Glu MTs varied substantially; however, it is clear that the nocodazole treatment decreased the number of Tyr MTs more rapidly than it decreased the number of Glu MTs. This is even more evident in Fig. 2 *A*, in which the data from three experiments such as the one shown in Table I have been presented graphically. At each time point of nocodazole treatment, we have plotted the average number of Tyr and Glu MTs that remained. The two curves show a 20–30% difference between the proportion of each type of MTs that remained at each time point. This difference was largely due to the rapid depolymerization of some Tyr MTs ( $\sim 50\%$  in the first 20 min); the remaining Tyr MTs depolymerized at an approximately constant rate, a rate equivalent to that characteristic of all Glu MTs.



**Figure 1.** Distribution of Tyr and Glu tubulin in nocodazole-treated TC-7 cells. Cells were treated with 0.2  $\mu\text{g/ml}$  nocodazole for the indicated times and then Triton-extracted, fixed, and stained with Tyr and Glu antibodies as described in Materials and Methods. Distribution of Tyr tubulin (*A*, *C*, and *E*) and Glu tubulin (*B*, *D*, and *F*) in cells before nocodazole treatment (*A* and *B*), after 15 min (*C* and *D*), and after 90 min (*E* and *F*) of nocodazole treatment. Bar, 5  $\mu\text{m}$ .

**Table 1. MT Depolymerization in Nocodazole-treated Cells**

Treatment time	MT type	Cell number									
		1	2	3	4	5	6	7	8	9	10
<i>min</i>											
0	Tyr	61	80	68	100	89	80	79	81	85	110
	Glu	3	4	14	32	32	22	29	31	39	28
15	Tyr	21	38	46	26	50	46	36	61	70	49
	Glu	0	37	28	6	18	32	15	20	0	35
90	Tyr	2	18	0	7	4	8	0	2	6	6
	Glu	3	20	0	7	3	8	0	2	8	4

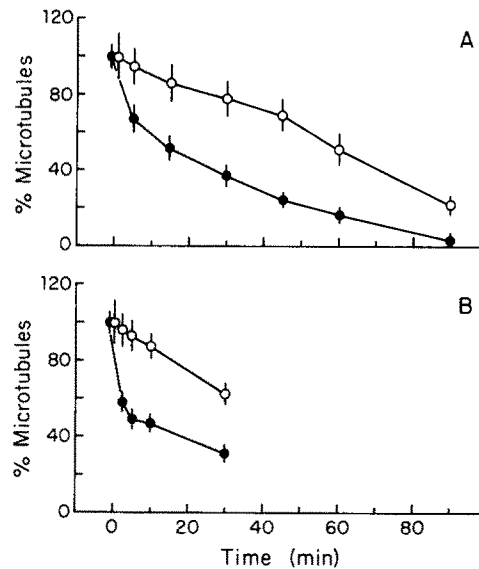
TC-7 cells were treated with nocodazole (0.2  $\mu\text{g/ml}$ ) as described in Materials and Methods. Tyr and Glu MT counts for 10 representative cells are shown for each of the treatment times that are shown in the immunofluorescence micrographs in Fig. 1.

In untreated cells, MTs stained only with the Tyr antibody comprise the largest subset of MTs (see Fig. 1, *A* and *B*; also, Gundersen et al., 1984, 1987a). However, after prolonged treatment with nocodazole, this class of MTs was almost completely absent (see Fig. 1, *E* and *F*, and Table I). In fact, at the 90-min timepoint 95% of the MTs that remained were stained with both antibodies. This is an important result because it suggests that Tyr MTs that possess a significant level of Glu subunits (i.e., those detectable with our Glu antibody) are more stable than those that do not have detectable Glu subunits.

The above experiments suggest that Glu MTs are more stable than Tyr MTs. However, there is an alternative explanation. Glu MTs are generated by postpolymerization detyrosination of Tyr MTs in as little as 20–25 min and all (Tyr) MTs appear to be susceptible to this conversion (Gundersen et al., 1987a). Thus, the stable Glu MTs we observed in treated cells could be attributable to either a population of stable Glu MTs that was present at the time of nocodazole addition, or to a stable population of Tyr MTs that was detyrosinated during the nocodazole treatment. To distinguish between these two possibilities, we treated cells with a higher concentration of nocodazole (0.6  $\mu\text{g/ml}$ ), so that MT depolymerization would proceed more quickly (Fig. 2 *B*). Tyr MT depolymerization was biphasic, as it had been with the lower concentration of nocodazole:  $\sim 50\%$  of Tyr MTs depolymerized rapidly while the remainder disassembled more slowly and at approximately the same rate as the Glu MTs. Most importantly, while 40% of the Tyr MTs were disassembled during a 2-min treatment, only 4% of the Glu MTs were depolymerized during this treatment. Since Glu MTs could not be created by detyrosination within such a short time, this result confirms the previous suggestion that Glu MTs are more stable to depolymerization by nocodazole than are Tyr MTs.

### ***In Vivo* Depolymerization of MT Subsets in Cold-treated Cells**

To determine if Glu MTs were more stable than Tyr MTs to other depolymerizing agents, we treated cells with cold (8°C) for various times. As shown in Fig. 3, quantification of the number of MTs remaining after various times of cold treatment showed that the rate of depolymerization of Tyr and Glu MTs did not differ significantly. A rapid decrease

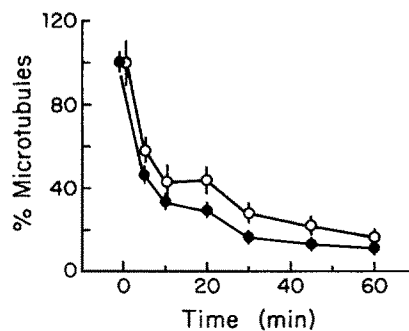


**Figure 2.** Time course of Tyr and Glu MT depolymerization by nocodazole treatment. TC-7 cells treated with 0.2 (*A*) or 0.6  $\mu\text{g/ml}$  nocodazole (*B*). The percentages of microtubules per cell that remain at each time are plotted: (●) Tyr MTs; (○) Glu MTs. Each determination represents the average number of MTs  $\pm$  SEM from 30 cells.

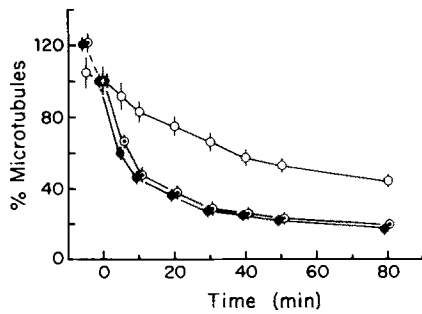
in both types of MTs was observed by 5 min of treatment, and this was followed by a slower decrease in which both Tyr and Glu MTs were lost at parallel rates. MTs in cold-treated cells showed more uniform behavior than those in drug-treated cells (compare errors in the measurements shown in Fig. 3 with those in Fig. 2). There was only a slight difference in the proportion of Tyr and Glu MTs remaining after 60 min of cold treatment (14 vs. 18%, respectively). These results suggest that there is no significant difference in the behavior of Tyr and Glu MTs toward cold-induced depolymerization.

### ***Stability of Tyr and Glu MTs to Dilution-induced Depolymerization***

As a third test of the stability of Tyr and Glu MTs, we detergent-extracted cells and then incubated the resultant cytoskeletons in PEM buffer (see Materials and Methods).



**Figure 3.** Time course of Tyr and Glu MT depolymerization by cold (8°C) treatment of TC-7 cells. The percentages of microtubules per cell that remain at each time point are plotted: (●) Tyr MTs; (○) Glu MTs. Each determination represents the average number of MTs  $\pm$  SEM from 30 cells.



**Figure 4.** Time course of Tyr, Glu, and exogenous Glu MT disassembly after extraction and dilution of the monomer pool. Some extracted cells were treated with CPA to generate Glu MTs (exogenous Glu MTs). TC-7 cells were Triton-extracted and then incubated in buffer to dilute the monomer pool. The percentages of microtubules per cell that remain at each time are plotted: (●) Tyr MTs; (○) Glu MTs; (◐) exogenous Glu MTs (MTs in CPA-treated cytoskeletons). Each determination represents the average number of MTs  $\pm$  SEM from 44 cells.

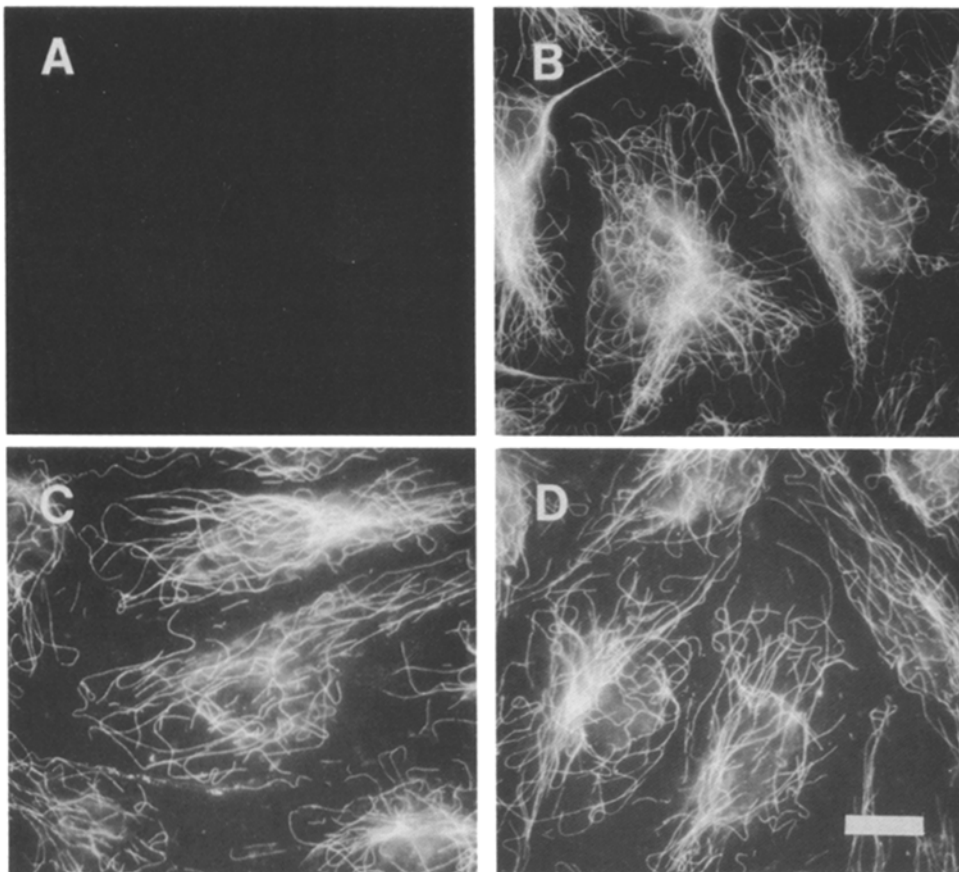
This induces depolymerization of MTs by dilution of the protomer pool. Quantification showed that there was a significant difference in the sensitivity of Tyr and Glu MTs to dilution (Fig. 4). A comparison of extracted and unextracted cells revealed that the extraction itself resulted in a decrease of  $\sim 20\%$  in the number of Tyr MTs, but no detectable decrease in Glu MTs. During the incubation of extracted cells, Tyr MTs disassembled rapidly, so that by 10 min after

extraction  $< 50\%$  of the Tyr MTs remained (Fig. 4). In contrast,  $< 20\%$  of the Glu MTs disassembled during the first 10 min of incubation (Fig. 4). With longer incubations, both types of MTs decreased in parallel, with  $\sim 25\%$  lost every 10 min.

As in nocodazole treatment there appeared to be two classes of Tyr MTs: labile ones that depolymerized more rapidly than Glu MTs, and more resistant ones that depolymerized with a rate similar to that of Glu MTs. Also, similar to the results from nocodazole treatment, the more resistant class of Tyr MTs was comparatively enriched in MTs that stained with both antibodies. Whereas the proportion of Tyr MTs stained with the Glu antibody was  $< 20\%$  in untreated cells, it was  $> 80\%$  in extracted cells incubated for 80 min. Unlike the Tyr MTs, Glu MTs appeared to depolymerize as one class, with a constant rate of loss throughout the dilution experiment.

#### Stability of Experimentally Generated Glu MTs

The experiments described above show that MTs containing Glu tubulin are more stable to nocodazole- and dilution-induced depolymerization. To test whether the stabilization of Glu MTs toward nocodazole and dilution was conferred by the presence of Glu subunits in the MTs, we created Glu MTs from the endogenous Tyr MTs by treating extracted cytoskeletons with CPA. CPA treatment results in the detyrosination of preexisting Tyr MTs in fixed cells (Gundersen et al., 1984) and we found that unfixed TC-7 cytoskeletons could be almost completely detyrosinated by CPA treat-



**Figure 5.** Distribution of Tyr and Glu tubulin in CPA-treated cytoskeletons. Cytoskeletons were prepared from TC-7 cells by Triton extraction and then incubated with (A, B, and D) or without CPA (C) for 5 min. Distribution of Tyr (A) and Glu (B) tubulin, showing the complete conversion of Tyr tubulin by the 5-min CPA treatment. Distribution of total tubulin in untreated (C) and CPA-treated (D) cytoskeletons after depolymerization was induced by dilution in buffer for 50 min. Bar, 5  $\mu$ m.

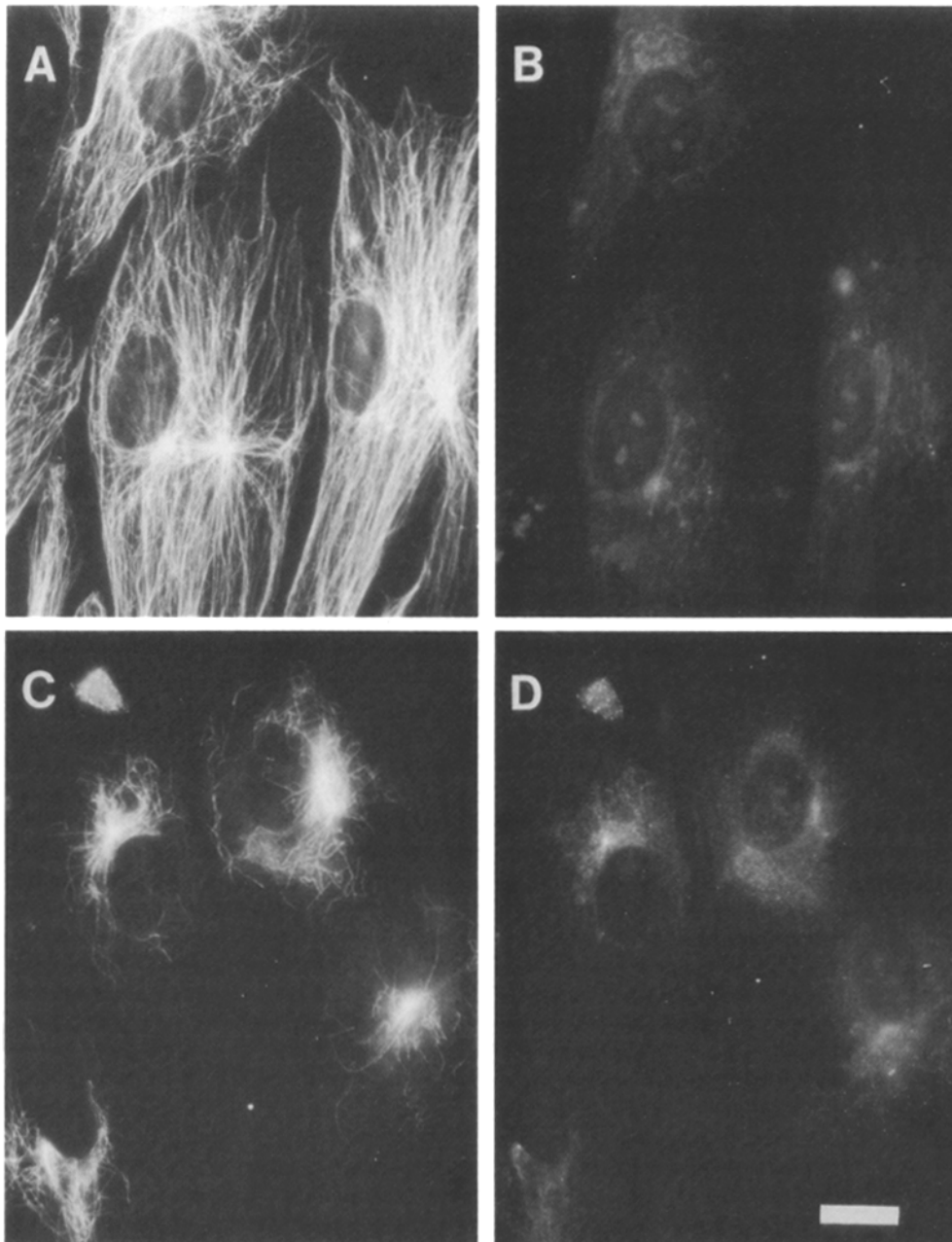
ment in as little as 5 min (compare Fig. 5, *A* and *B*, with Fig. 1, *A* and *B*). Immunoblots of CPA-digested cytoskeletons showed that Tyr tubulin comprised <10% of the total tubulin, while the level of Tyr tubulin in undigested cytoskeletons is  $\geq 90\%$  (Gundersen et al., 1987a). Thus, this brief CPA treatment generates MTs that, on the average, have greater than nine times as much Glu tubulin as the endogenous Tyr MTs. To distinguish Glu MTs created by CPA treatment from preexisting, endogenous Glu MTs, we will refer to the former as "exogenous Glu MTs."

Exogenous Glu MTs were indistinguishable from Tyr MTs in their resistance to dilution, as shown quantitatively in Fig. 4 and in the micrographs in Fig. 5, *C* and *D*. This is an important result since it suggests that factors other than the presence of Glu subunits within a MT are responsible for the

stabilization of endogenous Glu MTs. Because this experiment requires extraction of cells under conditions that remove endogenous MAPs (data not shown), our results do not rule out the possibility that a stabilizing MAP, specific for Glu tubulin, is responsible for enhancing the stability of endogenous Glu MTs (see Discussion).

#### *MT Stability in Cells that Do Not Contain Glu MTs*

Although most cultured cell lines and strains we have examined contain a population of Glu MTs, some have very few detectable Glu MTs. We examined one of these (the human fibroblast cell strain, 356) to determine if cells that lack Glu MTs contain stable MTs. These cells do contain Glu tubulin as judged by immunoblots (Webster et al., 1987a), yet only



**Figure 6.** Distribution of Tyr and Glu tubulin in nocodazole-treated 356 cells. Distribution of Tyr (*A* and *C*) and Glu (*B* and *D*) tubulin in cells before nocodazole treatment (*A* and *B*) and after 90 min of treatment with 0.3  $\mu\text{g/ml}$  nocodazole (*C* and *D*). Bar, 5  $\mu\text{m}$ .

an occasional cell has any MTs that contain a high enough level to be visualized using our immunofluorescence protocol (Fig. 6, A and B). In 356 cells treated with 0.3  $\mu\text{g}/\text{ml}$  of nocodazole most MTs depolymerized rapidly, but a small population of MTs in every cell was not depolymerized by even a 90-min nocodazole treatment (Fig. 6 C). As expected, these drug-stable MTs were not detectable with Glu antibody (Fig. 6 D). This result extends our analysis of the relationship of Glu level and MT stability: stability of a subset of cellular MTs neither requires nor results from a high level of Glu subunits.

## Discussion

The experiments reported in this paper demonstrate that, when compared with MTs enriched in Tyr tubulin (Tyr MTs), cellular MTs enriched in Glu tubulin (Glu MTs) possess a greater stability against depolymerization by nocodazole or dilution of the monomer pool. However, Glu MTs showed no enhanced stability relative to Tyr MTs in cold treatments. The enhanced stability of interphase Glu MTs is consistent with the correlation we described previously in differentiated cells (Gundersen and Bulinski, 1986a); Glu tubulin levels were elevated in the unusually stable MTs of axonemes, neurites, marginal bands, centrioles, and primary cells.

In evaluating the differences in MT stability that we have measured, it is important to consider sources of experimental errors, namely, in our counts of cellular MTs. We have counted single immunofluorescent fibers; however, many fibers may be too closely apposed to be resolved at the light microscopic level. This sort of error would serve to decrease our counts of Tyr MTs, especially in untreated cells or at early times in the depolymerizing treatment, since the dense array of straight Tyr MTs present near the centrosome is difficult to resolve into single fibers. The counts of Glu MTs would be expected to be a more accurate measure since fewer Glu MTs were present, and the sparse array of curly MTs could be resolved into distinct fibers quite readily. At later time points when few MTs remain, counts of both Glu and Tyr fibers would be expected to be accurate. In all of our counts, we have assumed that stained fibers correspond to single MTs rather than bundles of Glu or Tyr MTs; this assumption has been supported by electron microscopic studies in which no bundles of either Glu or Tyr MTs were observed (Geuens et al., 1986). An additional counting error stems from the protocol we used for fixation of the cells: To remove protomeric tubulin, which would have obscured the MTs, we extracted the cells with Triton X-100 before we fixed them. In untreated cells, this extraction markedly decreased the number of Tyr MTs, as compared with cells fixed without prior extraction; extraction had no effect on the number of Glu MTs present. Thus, both the dense packing of MTs and the extraction probably gave rise to an underestimate in the number of Tyr MTs at  $t = 0$  in each treatment. These errors alter our results only quantitatively; had errors in the Tyr MT counts been avoided, the difference in stability between Tyr and Glu MTs would have been even more dramatic than the difference we measured.

One possible explanation for the difference in stability we observed between Glu and Tyr MTs in vivo is that the high content of Glu tubulin in a MT could itself confer enhanced

stability. This explanation was rendered unlikely by our finding that the dilution stability of detergent-extracted cytoskeletons was identical whether or not the cytoskeletons were pretreated with carboxypeptidase A to substantially increase the Glu level in all MTs. Moreover, we determined that in human fibroblast cells in which no MTs possessed a high level of Glu tubulin, a significant population of MTs was resistant to drug treatment. These results are in agreement with previous in vitro comparisons of Glu/Tyr tubulin mixtures (Arce et al., 1978; Raybin and Flavin, 1976; Kumar and Flavin, 1982), supporting the contention that Glu tubulin content alone is neither necessary nor sufficient to stabilize MTs. Rather, high Glu tubulin content in a MT is more likely to be the result of stability conferred on that MT by another, as yet unknown, means.

Our finding that tubulin detyrosination does not have a direct effect on MT stability raises the question of what is causing the enhanced stability of this MT subset. Three mechanisms come to mind: alterations in the complement of MAPs (especially Glu-specific MAPs), other time-dependent post-translational modifications, or capping of MT ends. To date, no evidence of a heterogeneous distribution of MAPs exists in undifferentiated cells, though perhaps only the more prevalent (or universal) MAPs have thus far been studied. Glu MTs could be stabilized by Glu-specific- or -selective-binding proteins; however, no MAPs with this behavior have been described to date, and a subset of Tyr MTs (which presumably contain a low level of Glu tubulin and a correspondingly low level of any Glu-specific MAP) was also found to be stable in our study. Finally, MAPs are unlikely to be the only effectors of MT stability in vivo, since in our dilution experiment at least the predominant TC-7 cell MAP, primate 210K MAP (Bulinski and Borisy, 1980), was extracted, yet these cytoskeletal preparations still exhibited a stable MT subset.

Another posttranslational modification of tubulin, acetylation, might seem to be a good candidate for stabilizing MTs, though results demonstrating acetylation of tubulin in stable MT subsets (LeDizet and Piperno, 1986; Piperno et al., 1987) may reflect an effect rather than a cause of stability. We note, however, that early in the formation of MTs after release from nocodazole, small segments of acetylated tubulin appear on some MTs (Bulinski et al., 1988); these segments could be responsible for alterations in MT stability. Still, no definitive studies have examined the cause and effect relationship between tubulin acetylation and MT stability.

A final mechanism that could explain the stabilization of MTs is capping of the growing ends, such that subunit addition and loss are restricted. Though in interphase cultured cells nearly all MTs are either growing or shrinking (Soltys and Borisy, 1985; Schulze and Kirschner, 1986), a small subset of MTs does not appear to add or lose subunits for several hours (Schulze and Kirschner, 1987; Webster et al., 1987b). In other organisms, there is precedent for the capping of growing MT ends. For example, the central MTs in the ciliary axoneme of *Tetrahymena* are attached to a structure at the distal tip of the cilium (Dentler, 1984). In the mitotic spindle of cultured cells, MTs originating from the spindle pole (centrosome) can be capped at their growing ends by interaction with the kinetochore (Mitchison and Kirschner, 1985). Currently, there is no direct evidence that MTs of the interphase array of proliferating cells are capped or that they are attached to a regular structure such as the kinetochore.

Nonetheless, we believe that our data, as well as those obtained in other recent studies, are most consistent with the idea of MT capping in interphase cells. In our study we found that Glu MTs were more resistant to drugs or dilution, which are both thought to be end-mediated processes, but not to cold treatment. Mandelkow and Mandelkow (1985) have shown with cryoelectron microscopy that MTs subjected to temperatures  $\leq 10^{\circ}\text{C}$  were fragmented at sites along the wall of the MT, i.e., they did not exhibit depolymerization exclusively at their ends. Thus, the differential stability of Glu MTs to drugs or dilution, but not cold, can be explained by postulating caps on Glu MTs. Additional evidence for capping of Glu MTs is that Glu MTs do not add either endogenous or microinjected tubulin protomers to their distal ends, i.e., they are not growing (Gundersen et al., 1987b; Webster et al., 1987b). The inability of Glu MTs to add additional protomers, like the differential stability of Glu MTs, is most simply explained by the presence of a cap on the end of the MT.

It is important to make a distinction between the type of capping factor we envision, that is, one that interacts with the distal end of the MT to block further subunit addition or loss, and the previously described cap of GTP subunits (Carrier and Pantaloni, 1981; Hill and Carrier, 1983; Mitchison and Kirschner, 1984). If a GTP cap exists *in vivo*, it would be present on rapidly growing MTs (Tyr MTs), rather than on the nongrowing Glu MTs. Thus, end-mediated stabilization of Glu MTs probably does not involve a GTP cap.

In summary, we have shown that Glu MTs are more stable than Tyr MTs, and have shown that enrichment of Glu tubulin itself is insufficient to confer differential stability to MTs. We have not answered the question of how deetyrosination of MTs affects the behavior of MTs. The generation of Glu tubulin in MTs may permit the binding of specific MAPs; in this case, postpolymerization deetyrosination may serve as a signal of MT stability. Further studies will be necessary to determine the function of this posttranslational modification and to identify the factor(s) that contribute to the unusual stability of the Glu-enriched subset of cellular MTs.

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## References

- Arce, C. A., M. E. Hallak, J. A. Rodriguez, H. S. Barra, and R. Caputto. 1978. Capability of tubulin and microtubules to incorporate and to release tyrosine and phenylalanine and the effect of these amino acids on tubulin assembly. *J. Neurochem.* 31:205-210.
- Argarana, C. E., H. S. Barra, and R. Caputto. 1978. Release of [ $^{14}\text{C}$ ]-tyrosine from tubulinyl-tyrosine by brain extract. Separation of a carboxypeptidase from tubulin tyrosine ligase. *Mol. Cell Biochem.* 19:17-22.
- Argarana, C. E., H. S. Barra, and R. Caputto. 1980. Tubulinyl-tyrosine carboxypeptidase from chicken brain: properties and partial purification. *J. Neurochem.* 34:114-118.
- Barra, H. S., C. A. Arce, and R. Caputto. 1980. Total tubulin and its aminoacylated and nonaminoacylated forms during the development of rat brain. *Eur. J. Biochem.* 109:439-446.
- Barra, H. S., C. A. Arce, J. A. Rodriguez, and R. Caputto. 1973. A soluble preparation from rat brain that incorporates into its own proteins [ $^{14}\text{C}$ ]-

- arginine by a ribonuclease-sensitive system and [ $^{14}\text{C}$ ]-tyrosine by a ribonuclease-insensitive system. *J. Neurochem.* 20:97-108.
- Barra, H. S., C. A. Arce, J. A. Rodriguez, and R. Caputto. 1974. Some common properties of the protein that incorporates tyrosine as a single unit into microtubule proteins. *Biochem. Biophys. Res. Commun.* 60:1384-1390.
- Bulinski, J. C., and G. G. Borisov. 1980. Widespread distribution of a 210,000 mol wt microtubule-associated protein in cells and tissues of primates. *J. Cell Biol.* 87:802-808.
- Bulinski, J. C., J. E. Richards, and G. Piperno. 1988. Posttranslational modifications of  $\alpha$  tubulin: deetyrosination and acetylation differentiate populations of interphase microtubules in cultured cells. *J. Cell Biol.* In press.
- Carrier, M. F., and D. Pantaloni. 1981. Kinetic analysis of guanosine 5'-triphosphate hydrolysis associated with tubulin polymerization. *Biochemistry.* 20:1918-1924.
- Cassimeris, L. U., P. Wadsworth, and E. D. Salmon. 1986. Dynamics of microtubule depolymerization in monocytes. *J. Cell Biol.* 102:2023-2032.
- Deanin, G. G., W. C. Thompson, and M. W. Gordon. 1977. Tyrosyltubulin ligase activity in brain, skeletal muscle, and liver of the developing chick. *Dev. Biol.* 57:230-233.
- Dentler, W. L. 1984. Attachment of the cap to the central microtubules of *Tetrahymena* cilia. *J. Cell Sci.* 66:167-173.
- Flavin, M., and H. Murofushi. 1984. Tyrosine incorporation in tubulin. *Methods Enzymol.* 106:223-237.
- Forrest, G. L., and R. R. Klevecz. 1978. Tyrosyltubulin ligase and colchicine binding activity in synchronized Chinese hamster cells. *J. Cell Biol.* 78:441-450.
- Geuens, G., G. G. Gundersen, R. Nuydens, F. Cornelissen, J. C. Bulinski, and M. DeBrabander. 1986. Ultrastructural colocalization of tyrosinated and deetyrosinated alpha tubulin in interphase and mitotic cells. *J. Cell Biol.* 103:1883-1893.
- Gundersen, G. G., and J. C. Bulinski. 1986a. Microtubule arrays in differentiated cells contain elevated levels of a post-translationally modified form of tubulin. *Eur. J. Cell Biol.* 42:288-294.
- Gundersen, G. G., and J. C. Bulinski. 1986b. Distribution of tyrosinated and nontyrosinated alpha tubulin during mitosis. *J. Cell Biol.* 102:1118-1126.
- Gundersen, G. G., M. H. Kalnoski, and J. C. Bulinski. 1984. Distinct populations of microtubules: Tyrosinated and nontyrosinated alpha tubulin are distributed differently *in vivo*. *Cell.* 38:779-789.
- Gundersen, G. G., S. Khawaja, and J. C. Bulinski. 1987a. Postpolymerization deetyrosination of  $\alpha$ -tubulin: a mechanism for the subcellular differentiation of microtubules. *J. Cell Biol.* 105:251-264.
- Gundersen, G. G., S. Khawaja, and J. C. Bulinski. 1987b. Microtubules enriched in deetyrosinated tubulin *in vivo* are less dynamic than those enriched in tyrosinated tubulin. In *The Cytoskeleton in Cell Differentiation and Development*. R. B. Maccioni and J. Arechaga, editors. ICSU Press, Paris. In press.
- Hill, T. L., and M. F. Carrier. 1983. Steady-state theory of the interference of GTP hydrolysis in the mechanism of microtubule assembly. *Proc. Natl. Acad. Sci. USA.* 80:7234-7238.
- Kilmartin, J. V., B. Wright, and C. Milstein. 1982. Rat monoclonal antitubulin antibodies derived by using a new nonsecreting rat cell line. *J. Cell Biol.* 93:576-582.
- Kumar, N., and M. Flavin. 1981. Preferential action of a brain deetyrosinating carboxypeptidase on polymerized tubulin. *J. Biol. Chem.* 256:7678-7686.
- Kumar, N., and M. Flavin. 1982. Modulation of some parameters of assembly of microtubules *in vitro* by tyrosination of tubulin. *Eur. J. Biochem.* 128:215-222.
- LeDizet, M., and G. Piperno. 1986. Cytoplasmic microtubules containing acetylated  $\alpha$ -tubulin in *Chlamydomonas reinhardtii*: spatial arrangement and properties. *J. Cell Biol.* 103:13-22.
- Mandelkow, E. M., and E. R. Mandelkow. 1985. Unstained microtubules studied by cryo-electron microscopy: substructure, supertwist, and disassembly. *J. Mol. Biol.* 181:123-135.
- Mitchison, T. J., and M. W. Kirschner. 1984. Dynamic instability of microtubule growth. *Nature (Lond.)* 312:237-242.
- Mitchison, T. J., and M. W. Kirschner. 1985. Properties of the kinetochore *in vitro*. III. Microtubule capture and ATP-dependent translocation. *J. Cell Biol.* 101:766-777.
- Moyer, S. A., S. C. Baker, and J. L. Lessard. 1986. Tubulin: a factor necessary for the synthesis of both Sendai virus and vesicular stomatitis virus RNAs. *Proc. Natl. Acad. Sci. USA.* 83:5405-5409.
- Nath, J., and M. Flavin. 1979. Tubulin tyrosylation *in vivo* and changes accompanying differentiation of cultured neuroblastoma-glioma hybrid cells. *J. Biol. Chem.* 254:11505-11510.
- Nath, J., M. Flavin, and J. I. Gallin. 1982. Tubulin tyrosination in human polymorphonuclear leukocytes: studies in normal subjects and in patients with the Chediak-Higashi syndrome. *J. Cell Biol.* 95:519-526.
- Piperno, G., M. LeDizet, and X. Chang. 1987. Microtubules containing acetylated  $\alpha$ -tubulin in mammalian cells in culture. *J. Cell Biol.* 104:289-302.
- Raybin, D., and M. Flavin. 1977a. Enzyme which specifically adds tyrosine to the  $\alpha$ -chain of tubulin. *Biochemistry.* 16:2189-2194.
- Raybin, D., and M. Flavin. 1977b. Modification of tubulin by tyrosylation in cells and extracts and its effects on assembly *in vitro*. *J. Cell Biol.* 73:492-504.



- Rodriguez, J. A., and G. G. Borisy. 1978. Modification of the C-terminus of brain tubulin during development. *Biochem. Biophys. Res. Commun.* 83: 579-586.
- Rodriguez, J. A., and G. G. Borisy. 1979. Tyrosination state of free tubulin subunits and tubulin disassembled from microtubules of rat brain tissue. *Biochem. Biophys. Res. Commun.* 89:893-899.
- Schulze, E., and M. Kirschner. 1986. Microtubule dynamics in interphase cells. *J. Cell Biol.* 102:1020-1031.
- Schulze, E., and M. Kirschner. 1987. Dynamic and stable populations of microtubules in cells. *J. Cell Biol.* 104:277-288.
- Soltys, B., and G. G. Borisy. 1985. Polymerization of tubulin in vivo: direct evidence for assembly onto microtubule ends and from centrosomes. *J. Cell Biol.* 100:1682-1689.
- Thompson, W. C., D. J. Asai, and D. H. Carney. 1984. Heterogeneity among microtubules of the cytoplasmic microtubule complex detected by a monoclonal antibody to alpha tubulin. *J. Cell Biol.* 98:1017-1025.
- Webster, D. R., G. G. Gundersen, J. C. Bulinski, and G. G. Borisy. 1987a. Assembly and turnover of detyrosinated tubulin in vivo. *J. Cell Biol.* 105: 265-276.
- Webster, D. R., G. G. Gundersen, J. C. Bulinski, and G. G. Borisy. 1987b. Differential turnover of tyrosinated and detyrosinated microtubules. *Proc. Natl. Acad. Sci. USA.* In press.
- Wehland, J., M. C. Willingham, and I. V. Sandoval. 1983. A rat monoclonal antibody reacting with the tyrosylated form of  $\alpha$ -tubulin. I. Biochemical characterization, effects on microtubule polymerization *in vitro*, and microtubule polymerization and organization *in vivo*. *J. Cell Biol.* 97:1467-1475.