

# Detyrosination of Alpha Tubulin Does Not Stabilize Microtubules In Vivo

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**Abstract.** The relationship between alpha tubulin detyrosination and microtubule (MT) stability was examined directly in cultured fibroblasts by experimentally converting the predominantly tyrosinated MT array to a detyrosinated (Glu) array and then assaying MT stability. MTs in mouse Swiss 3T3 cells displayed an increase in Glu immunostaining fluorescence  $\sim 1$  h after microinjecting antibodies to the tyrosinating enzyme, tubulin tyrosine ligase. Detyrosination progressed to virtual completion after 12 h and persisted for 30–35 h before tyrosinated subunits within MTs were again detected. The stability of these experimentally detyrosinated MTs was tested by first injecting either biotinylated or Xrhodamine-labeled tubulin and then measuring bulk turnover by hapten-mediated immunocytochemistry or fluorescence recovery after photobleaching, respectively. By both

methods, turnover was found to be similarly rapid, possessing a half time of  $\sim 3$  min. As a final test of MT stability, the level of acetylated tubulin staining in antibody-injected cells was compared with that observed in adjacent, uninjected cells and also with the staining observed in cells whose MTs had been stabilized with taxol. Although intense Glu staining was observed in both injected and taxol-treated cells, increased acetylated tubulin staining was observed only in the taxol-stabilized MTs, indicating that the MTs were not stabilized by detyrosination. Together, these results demonstrated clearly that detyrosination does not directly confer stability on MTs. Therefore, the stable MTs observed in these and other cell lines must have arisen by another mechanism, and may have become posttranslationally modified after their stabilization.

**T**HE mechanism(s) underlying microtubule (MT)<sup>1</sup> function may exploit its biological characteristics. A hallmark of MTs is their dynamic nature, whereby individuals lengthen and shorten rapidly (Mitchison and Kirschner, 1984; Cassimeris et al., 1988), and hundreds may turn over within minutes (Saxton et al., 1984; Schulze and Kirschner, 1986; Sammak et al., 1987). The continual rearrangement of MTs (or MT ends) that arises as a direct consequence of rapid turnover may be particularly useful in accommodating the cell-shape changes associated with morphogenesis and cell motility (Kirschner and Mitchison, 1986; Sammak and Borisy, 1988b). However, some individual MTs as well as some MT assemblies are extremely stable and turn over with half times of hours, not minutes (Webster et al., 1987b; Webster and Borisy, 1989; Lim et al., 1989). In addition,

some MT-containing organelles including the axonal core of neurites and the mitotic apparatus contain both dynamic and stable MTs (Sahenk and Brady, 1987; Lim et al., 1989; Brinkley and Cartwright, 1975; Gorbsky and Borisy, 1989), demonstrating an apparent requirement for both types of MTs for proper organelle function and a mechanism for endowing particular MTs with increased stability.

Individual MTs might become differentially stabilized by their binding to specific associated proteins or by the use of particular genetic isoforms of the tubulin subunits. However, the posttranslational modification of MTs would offer the cell a rapid means of stabilizing individual MTs in response to various intra- or extracellular signals. Stable MTs in cultured cells and in cells examined in situ have often been found to contain increased levels of posttranslationally modified subunits (L'Hernault and Rosenbaum, 1983; Black and Keyser, 1987; Gundersen and Bulinski, 1986; Schulze et al., 1987; Webster et al., 1987b; Wehland and Weber, 1987b; Webster and Borisy, 1989). Alpha tubulin may be reversibly detyrosinated at its COOH-terminus (Arce et al., 1978) or acetylated at Lys40 (LeDizet and Piperno, 1987), while at least one beta tubulin isotype may be reversibly phosphorylated at a serine residue that is situated near the COOH-terminus (Gard and Kirschner, 1985; Serrano et al.,

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1. *Abbreviations used in this paper:* Ac, acetylated; Glu, detyrosinated; MAP, microtubule-associated protein; MT, microtubule; TTL, tubulin tyrosine ligase; Tyr, tyrosinated.

1987). The detyrosination, acetylation, and phosphorylation reactions occur primarily on tubulin assembled into MTs (Kumar and Flavin, 1981; LHernault and Rosenbaum, 1983; Black et al., 1989; Gard and Kirschner, 1985), while the complementary reactions occur primarily on unassembled dimers (Raybin and Flavin, 1975; LHernault and Rosenbaum, 1985; Gard and Kirschner, 1985). Although the restriction of each enzyme activity to one phase of the tubulin assembly cycle suggests a functional relationship between them as well, the relationship between posttranslational modification and MT function (including their increased stability) remains uncertain. However, evidence has recently been obtained for the association of stable detyrosinated (Glu) MTs with the development of cellular asymmetries that are established during the initiation of directed cell locomotion (Gundersen and Bulinski, 1988).

The rate and extent of assembly of Glu and "maximally tyrosinated" (Tyr) brain tubulin, as measured *in vitro* on bulk samples of MT protein (tubulin plus added saturating levels of MT-associated proteins [MAPs] or MAP 2), were found to be indistinguishable (Arce et al., 1978; Raybin and Flavin, 1977; Kumar and Flavin, 1982). One study (Kumar and Flavin, 1982) reported that Tyr MTs polymerized faster than Glu MTs in the presence of subsaturating levels of taxol but indistinguishably from Glu MTs when saturating amounts were used, suggesting that some difference in the assembly competence of the two tubulin forms may exist. A more recent study (Webster et al., 1987a), in which brain Glu tubulin was microinjected into fibroblasts, revealed incorporation onto the ends of all discernable MTs and later into the bulk of the array, in a manner indistinguishable from untreated (but hapten-labeled) brain tubulin. However, the level of Glu subunits incorporated into MTs (~10–20%) may have been too low to affect the assembly properties of the entire network.

Although some evidence implied that the posttranslational modification of tubulin arose as a consequence of MT stability (Webster et al., 1987b; Webster and Borisy, 1989; Khawaja et al., 1988), no direct tests of this relationship could be performed in living cells, since reagents that could specifically disrupt either cycle were unavailable. Recently, antibodies to the tyrosinating enzyme, tubulin tyrosine ligase (TTL), were developed that inhibited its activity both *in vitro* (Wehland and Weber, 1987a) and *in vivo* (Wehland and Weber, 1987b). Although the TTL activity was inhibited in antibody-injected cells the detyrosinating enzyme activity remained uninhibited, resulting over time in MTs that became progressively detyrosinated (Wehland and Weber, 1987b). We sought to test the relationship between tubulin detyrosination and MT stabilization directly by microinjecting the TTL antibodies into fibroblasts, incubating the cells until the MT array had become maximally detyrosinated, and then assaying MT stability by three independent methods. The results of these tests demonstrated conclusively that detyrosination does not directly confer stability on MTs, and therefore stabilization must arise by a different mechanism.

## Materials and Methods

### Cell Lines and Reagents

Mouse Swiss 3T3 fibroblasts (ATCC #CCL 92) were maintained in Ham's

F-10 medium supplemented with 10% (vol/vol) calf serum. Biotinylated tubulin (Webster and Borisy, 1989) and Xrhodamine-labeled tubulin (Sammak and Borisy, 1988a) were prepared as previously described. The polyclonal and monoclonal antibodies to TTL as well as the Glu (IgG) and tyrosinated (Tyr) IgM tubulin antibodies were produced and described elsewhere (Wehland and Weber, 1987a,b). The antibody to Ac tubulin was a kind gift from Dr. Gianni Piperno, Rockefeller University (New York). Ethylene glycol bis-(succinic acid *N*-hydroxysuccinimide ester) (EGS) was obtained from Sigma Chemical Co. (St. Louis, MO), and the Texas Red-conjugated streptavidin and all secondary antibodies were purchased from Jackson Immunoresearch Laboratories Inc. (Avondale, PA).

### Serum Deprivation

Cells to be analyzed for their conversion to Glu or the stability of their MTs were first incubated for 24 h in medium lacking serum. The medium overlying coverslips in individual 35-mm dishes was aspirated off, washed once with medium lacking serum, and replaced with serum-free medium. To assess the possible effects of serum deprivation and the two microinjection procedures on cell health, growth curves of uninjected control populations were compared either with cells treated only by serum deprivation or with cells serum deprived and then microinjected twice. The most significant effect on growth was noted after the 24 h of serum deprivation; cells ceased to grow on any coverslip and in some cases the cell number declined slightly. However, the cells that survived this treatment were not further affected by the subsequent microinjections; those cells began growing exponentially at a rate comparable to that of the uninjected cells on the same coverslips and to that of control populations.

### Conversion Kinetics and Ac Tubulin Immunostaining

All of the serum-deprived cells within a cell-free zone were microinjected with either the polyclonal or the monoclonal TTL antibodies and incubated for 1–72 h. At that time the cells were rinsed once in warm PHEM buffer (60 mM Pipes, pH 6.95, 25 mM HEPES, 10 mM EGTA, and 2 mM MgCl<sub>2</sub>) (Schliwa et al., 1981), lysed for 1 min with PHEM containing 0.1% (vol/vol) Triton X-100, and then fixed for 20 min in PHEM containing 5 mM EGS. Cells were immunostained with Glu IgG and Tyr IgM antibodies and fluorescein and Texas Red-conjugated secondary antibodies specific for mouse IgG and IgM, respectively. For the observation of Ac tubulin levels in cells previously injected with the polyclonal TTL antibodies, cells were first serum deprived and incubated for 24 h after injection, then rinsed in warm PHEM buffer, and immersed immediately in ice-cold methanol containing 5 mM EGTA. Coverslips were then immunostained with Texas Red-conjugated goat anti-rabbit antibodies and either Glu or Ac tubulin antibodies followed by fluorescein-conjugated goat anti-mouse antibodies. Other immunofluorescence and photographic procedures and equipment that were used have been described (Webster and Borisy, 1989).

### Analysis of MT Turnover

All of the serum-deprived cells within a defined area were microinjected with the TTL antibodies, incubated for 24 h, and then selected cells were injected with biotinylated tubulin. Cells incubated for <2, 10, or 60 min were rinsed with warm PHEM buffer, lysed for 1 min, and then fixed with EGS. Cells were stained sequentially with Texas Red-conjugated streptavidin, Glu antibodies, and fluorescein-conjugated, goat anti-mouse antibodies. Photographic prints were traced into clear acetate (Webster et al., 1987a) and the proportions of Glu MTs that had or had not turned over were tabulated for each time interval. The mean values  $\pm$  SD were weighted as described (Webster and Borisy, 1989).

Alternatively, serum-deprived cells were first injected with Xrhodamine-labeled tubulin, incubated for 1–2 h, and then located by scanning the coverslip at low magnification using epi-illumination and a rhodamine filter set. These cells were injected also with TTL antibodies, incubated 14–30 h, and subjected to photobleaching experiments. The photobleaching procedure and the apparatus that was used have been described extensively elsewhere (Sammak and Borisy, 1988a). Briefly, double-injected cells were located on the coverslip by their fluorescence, aligned on the microscope stage to be oriented with their long axes perpendicular to the bleaching beam, and bleached using a 514-nm, 200-MW beam for 100 ms, corresponding to a beam strength of 20 mW/m<sup>2</sup>. Photobleached cells were monitored for recovery of fluorescence using a cooled charge-coupled device camera (model 200; Photometrics Ltd., Tucson, AZ), and phase and fluorescence images were recorded on both a video frame recorder (model P-61U; Mitsubishi Electronic Sales America, Inc., Rancho Dominguez, CA) and a WORM

drive optical disc recorder (type 3363; IBM Corp., Danbury, CT) for later analysis. Cells were also monitored for any changes in phase density, MT arrangement, or cell morphology that would indicate declining cell health. Photodissolution and photostabilization effects (Vigers et al., 1988) were judged to be minimal, for the following reasons. First, the total exposure of the cells to the bleaching beam was much less than in the cited study. Also, bleached cells never lost their fibrous staining outside of the bleached zones, presumably due both to the mild photobleaching conditions used and the low-light CCD camera equipment used to capture the images. Finally, the turnover rates observed for both the TTL antibody-injected cells and the controls in the photobleaching experiments were similar to the turnover rates calculated from the immunocytochemistry experiments. Although photodissolution effects were not studied in detail and therefore could not be ruled out altogether, their contributions to the results presented here were clearly not large.

## Results

### Experimental Approach

Cellular tyrosinated (Tyr) MTs were experimentally converted to Glu MTs and then the stability of the Glu array was tested. Swiss 3T3 fibroblasts were first microinjected with antibodies to TTL. After the conversion from Tyr to Glu was essentially complete MT stability was examined in three ways. First, biotinylated tubulin was injected and immunocytochemistry was performed on lysed and fixed cells in order to map the sites of subunit incorporation and to determine the kinetics of full incorporation into the experimentally altered cytoskeleton. Second, fluorescently derivatized tubulin was injected and photobleaching experiments were performed on live cells in order to analyze Glu MT turnover under conditions where injection perturbation was minimal. Third, the level of Ac tubulin staining on converted Glu MTs was determined. Acetylation occurs at a distinct site from detyrosination on alpha tubulin (LeDizet and Piperno, 1987) and has been correlated with stable MTs in vivo (Piperno et al., 1987; Webster and Borisy, 1989), and therefore offered an independent measure of MT stability. By these assays, the relationship between detyrosination and MT stability was directly examined.

Many tissue culture cell lines possess an extensive Tyr-staining MT array (Gundersen et al., 1984; Webster et al., 1987a,b; Wehland and Weber, 1987b), due to an active TTL (Gundersen et al., 1987; Webster et al., 1987a). Since proliferating monolayers of cells display more Glu MTs after reaching confluence (Webster et al., 1987a), after incubation with cAMP analogues, or after incubation without serum (Wehland and Weber, 1987b), cells to be injected with the TTL antibody were first incubated without serum for 24 h, in order to temporarily induce a more "differentiated" state similar to that induced in neuronal cells (Amano et al., 1972) and thereby increase the effectiveness of the injected antibody. Cells treated in this manner flattened out on the coverslip and mitotic cells were rarely observed. Some cells retracted their edges and floated off of the coverslip; however, many retained their flattened morphology throughout the 24 h of incubation. Although immunostaining for Ac subunits perhaps increased slightly, the level of Glu staining remained unchanged after this treatment. Cells released from serum deprivation for 24 h grew exponentially and displayed MT dynamics indistinguishable from serum-fed cells, as assayed by incorporation of hapten-labeled tubulin into MTs (data not shown). Experimental cells were injected af-

ter 24 h of serum deprivation with either an affinity-purified polyclonal antibody (~3 mg/ml) or a monoclonal antibody purified from ascites fluid (~3 mg/ml) specific for TTL and then released into serum-containing media for further incubation. Each antibody type gave indistinguishable results and was used interchangeably; however, the effects of both antibodies were enhanced, as compared with the serum-fed controls, when injected after the cells had undergone serum deprivation.

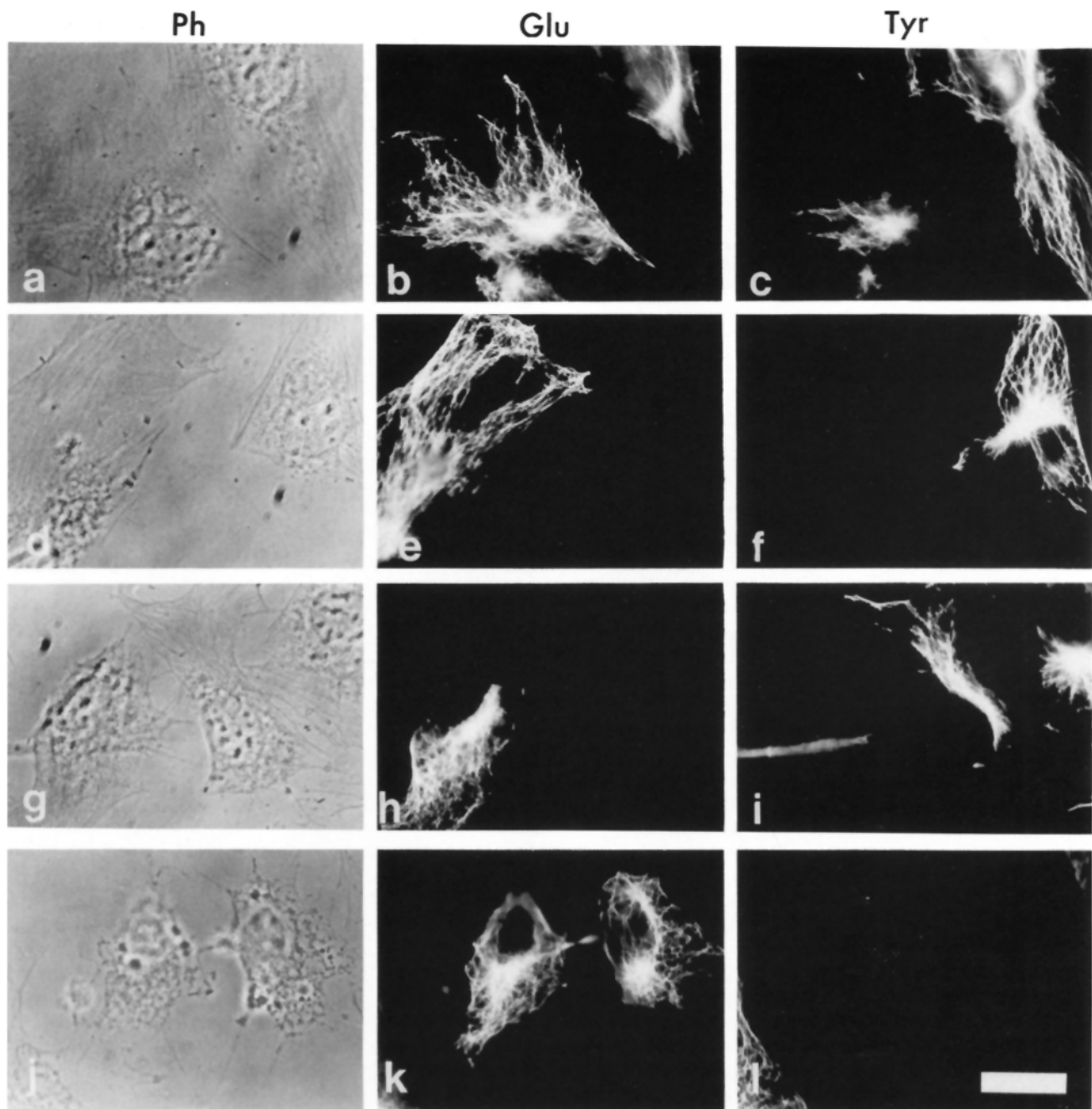
### Kinetics and Persistence of Experimentally-produced Glu MTs

Swiss 3T3 cells were injected with either of the TTL antibody preparations and then incubated 1-72 h in order to establish the time interval of maximal Tyr to Glu conversion. Fig. 1 shows the progression of detyrosination after the polyclonal antibody was injected. In each case only one of two closely apposed cells was injected leaving the other for the comparison of steady-state Glu and Tyr staining levels. By capturing both cells in one photographic negative the Glu and Tyr staining intensities of the two cells could be directly compared.

A significant rise in the Glu staining of MTs was observed after 1-2 h of incubation with the injected antibody. After 6 h of incubation the Glu staining became brighter than the Tyr staining (Fig. 1, *b* and *c*; compare the Glu and Tyr images of the injected cell on the left in each panel with those of the uninjected cell on the right). The level of Tyr staining in the injected cell, although still above background, was much reduced as compared with either the intensity of Glu staining found in the same cell or the intensity of Tyr staining observed in its uninjected counterpart (Fig. 1 *c*). The accumulation of Glu subunits appeared evenly distributed among all MTs of the cytoskeleton, in contrast to the lack of Glu staining usually observed or the small subset of sinuous Glu MTs that were occasionally observed in untreated cells. No changes in cell shape or other signs of cell damage were apparent after the serum deprivation and microinjection procedures were performed (Fig. 1 *a*), confirming that the increase in Glu staining was not a result of declining cell health.

By 8-12 h after TTL antibody injection, the conversion from Tyr to Glu MTs was essentially complete (Fig. 1, *e* and *f*). The Glu staining intensity remained high in injected cells (Fig. 1 *e*), but the corresponding Tyr staining was virtually absent (Fig. 1 *f*). No other changes in the cytoskeleton were observed, including, for example, the redistribution of MTs or a change in morphology from straight to curly MTs, indicating that detyrosination does not directly determine the appearance of Glu MTs in untreated cells (Gundersen et al., 1984; Wehland and Weber, 1987b). The intense Glu staining as well as the low level of Tyr staining was still evident after 24 h of incubation (Fig. 1, *g-i*).

Some injected cells that were subsequently incubated for 12-24 h had entered or completed mitosis at the time of cell lysis (Fig. 1, *j-l*). Although the level of injected TTL antibody was probably reduced by 50% in the daughter cells, the level of Glu subunits in the newly formed interphase arrays remained high while the Tyr staining remained negligible. This retention of Glu surpasses the longevity of the cell's stable Glu subset (Webster et al., 1987b), which is broken down during the two global MT transformations that occur



**Figure 1.** Tubulin tyrosine ligase inhibition quantitatively converts Tyr MTs to Glu MTs. Swiss 3T3 cells were cultured without serum for 24 h and then one of a closely spaced pair was selected for microinjection with the polyclonal TTL antiserum (the injected cells are on the left in all panels). The cells were then incubated for 6, 12, or 24 h before they were lysed, fixed, and double immunostained for the presence of Glu and Tyr tubulin in MTs. The phase-contrast micrographs (*a, d, g, j*) show the position of all cells in the fields of view. After 6 h of incubation, the Glu staining of the injected cells had risen dramatically as compared with their uninjected neighbors (*b*), while the Tyr staining of the injected cells had become diminished (*c*). The immunofluorescence signal appeared uniformly bright over all MTs of the cytoskeleton. After 12 h the Glu staining in the injected cells remained high (*e*); however, by this time the Tyr staining had dropped below the threshold of visibility (*f*). The high Glu and low Tyr levels persisted in interphase cells for 24 h (and longer) after injection (*h* and *i*) while other injected cells proceeded through cell division using only Glu subunits (*j-l*). The cell in *j-l* was incubated for 12 h after injection. Bar, 20  $\mu\text{m}$ .

during prophase and late telophase. Indeed, cells incubated for >40 h (two to three cell generations) after TTL antibody injection still exhibited a high level of Glu staining, although by that time the level of Tyr staining had begun to rise (data not shown). The injection of the TTL antibody shifted the

predominantly Tyr array to a predominantly Glu MT array but did not prevent the cells from dividing, nor did it alter other cellular properties including saltatory particle movement, the distribution of vimentin type intermediate filaments, or cell motility (data not shown).

The rate of Tyr to Glu conversion was also studied in other cell lines, including a primary human fibroblast cell line (type 356), which usually does not contain a Glu MT array, African green monkey kidney epithelial cells (line TC-7), which normally contain many Glu MTs, and porcine kidney epithelial cells (line LLC-PK<sub>1</sub>), which contain a moderate number of Glu MTs. The 356 cells and the LLC-PK<sub>1</sub> cells demonstrated conversion kinetics that were similar to that found for 3T3 cells. However, a significant elevation of Glu staining was evident in the TC-7 line after as little as 30 min of incubation, suggesting that TC-7 cells may have possessed a greater tubulin carboxypeptidase activity than was present in the other cell lines.

### Converted Glu MTs Turn Over Rapidly

Biotinylated tubulin that was injected into TTL antibody-injected cells became incorporated rapidly into the Glu array (Fig. 2). Within 2 min after the second (tubulin) injection, biotin staining was observed at the ends of many Glu MTs (arrowheads in Fig. 2, *a* and *b*). However, some Glu MT ends were not biotinylated, suggesting that they were not growing (asterisk in Fig. 2, *a* and *b*; also note the lack of biotin staining on MT ends near the right arrowhead). After 10 min of incubation (Fig. 2, *c* and *d*) biotinylated subunits had become uniformly incorporated into many MTs (arrow in Fig. 2 *c*); however, some MTs still lacked hapten (asterisks in Fig. 2, *c* and *d*). By 1 h after the tubulin injection, virtually all MTs were colabeled for hapten and Glu (Fig. 2, *e* and *f*), demonstrating that the experimentally detyrosinated MTs in these cells had turned over rapidly. The quantified results of this analysis are given in Table I. In this study, 77% of the Glu MT ends displayed growth zones containing biotin. In previous studies, however, virtually all Tyr MTs supported the end growth of injected subunits, whether the injectant contained predominantly Tyr subunits (Webster et al., 1987*b*), Glu subunits (Webster et al., 1987*a*), or a mixture of both (Soltys and Borisy, 1985; Schulze and Kirschner, 1986). The decrease in the proportion of growing Glu ends observed in this study may be at least partly explained by the presence of a stable (and presumably nongrowing) Glu subset in these cells (Wehland and Weber, 1987*b*), whose MT ends would be indistinguishable from the dynamic Glu MT ends by our immunofluorescence assay. Alternatively, the proportions of growing (77%) and shrinking (23%) Glu MTs closely match the estimated proportions of growing (80%) and shrinking (20%) MTs studied *in vitro* using purified tubulin (Mitchison and Kirschner, 1984). Whether or not these *in vivo* Glu MTs mimicked the behavior of the *in vitro* MTs is unclear. However, it seems unlikely that, in light of the dynamic behavior exhibited by these Glu MTs, the absence of biotinylated segments at the ends of a minor proportion was due to their stabilization by detyrosination.

10 min after the injection of hapten-labeled tubulin 90% of the Glu MTs were colabeled, corresponding to a turnover half time of ~3 min. This value is somewhat less than the half times calculated for Tyr MT turnover (5–20 min, Saxton et al., 1984; Schulze and Kirschner, 1986; Sammak et al., 1987; Webster et al., 1987*a*) and may perhaps represent a real difference as compared with Tyr MT dynamics *in vivo*. After 1 h of incubation, all MTs were copolymers of biotinylated and Glu subunits, confirming the dynamic nature

of the Glu network. These results demonstrated that the experimentally derived Glu MTs turned over rapidly, with kinetics similar to those of Tyr MTs but in contrast to the dynamics characteristic of sinuous Glu MTs, some of which were found to be stable for most of the cell cycle (Webster et al., 1987*b*).

### Photobleached Glu MTs Are Not Stable

MT stability was also tested by photobleaching Xrhodamine-labeled MTs in a plane perpendicular to the leading edge of the cell, and then comparing the intervals required for full fluorescence recovery in antibody-injected and control cells. The cell in Fig. 3 was photobleached twice within ~20 s: first 9.4  $\mu$ m and then 18  $\mu$ m from the leading edge. By visual examination of video prints the bleached regions recovered full fluorescence intensity simultaneously, requiring <5 min from the time that the second bleaching pulse was delivered. The average recovery time ( $\pm$  SD) for the antibody-injected cells (10 bleached regions from six cells) was 7 min, 21 s  $\pm$  2 min, 17 s, and for the control cells injected with only Xrhodamine tubulin (13 bleached regions from six cells), 9 min, 43 s  $\pm$  3 min, 47 s. Although the mean recovery time for the antibody-injected cells was somewhat shorter than for the control cells, the values were not statistically different. The results of this test clearly showed that the experimentally converted Glu MTs were not stabilized by their detyrosination, and were perhaps slightly more dynamic than the usually predominant Tyr MT array. Human fibroblasts that were similarly bleached gave virtually identical results.

### Experimentally Produced Glu MTs Are Not Acetylated

The stability of experimentally detyrosinated MTs was qualitatively examined by comparing the levels of Ac subunits in injected cells with those found in adjacent, uninjected cells. Serum-deprived Swiss 3T3 cells were injected with the TTL antibodies, incubated for 24 h, fixed with ice-cold methanol, and then immunostained for the TTL antibody to identify the injected cells and either Glu or Ac tubulin to detect modified MTs (Fig. 4). Fig. 4, *a-c* demonstrates that the MT network observed in antibody-injected cells (Fig. 4 *b*) was enriched with Glu subunits (Fig. 4 *c*) while neighboring uninjected

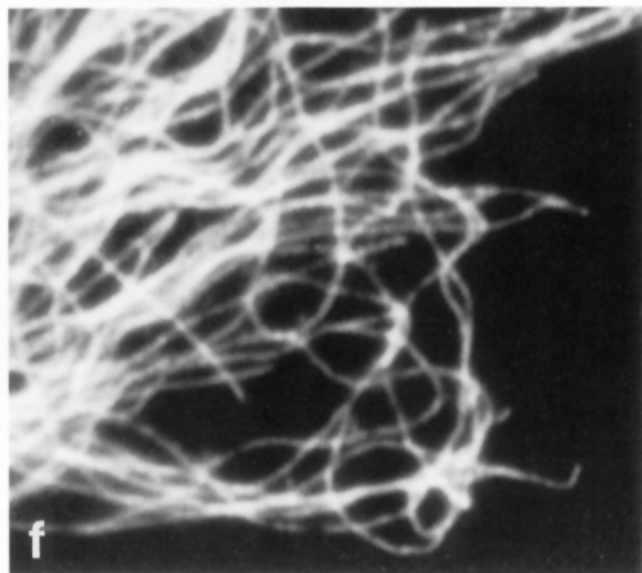
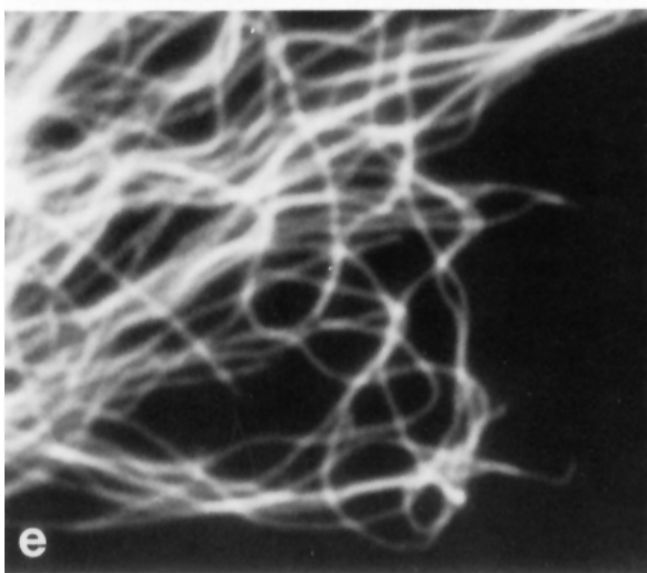
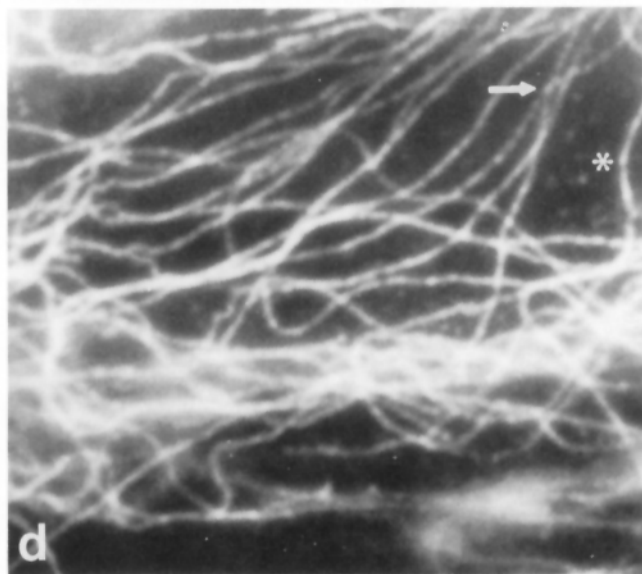
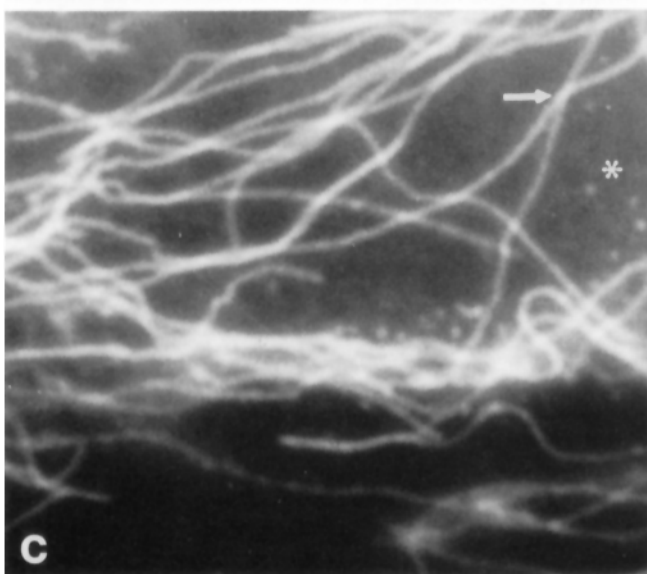
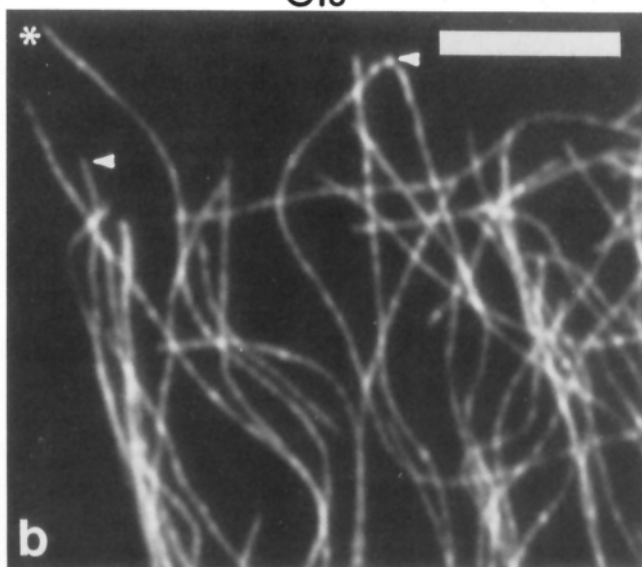
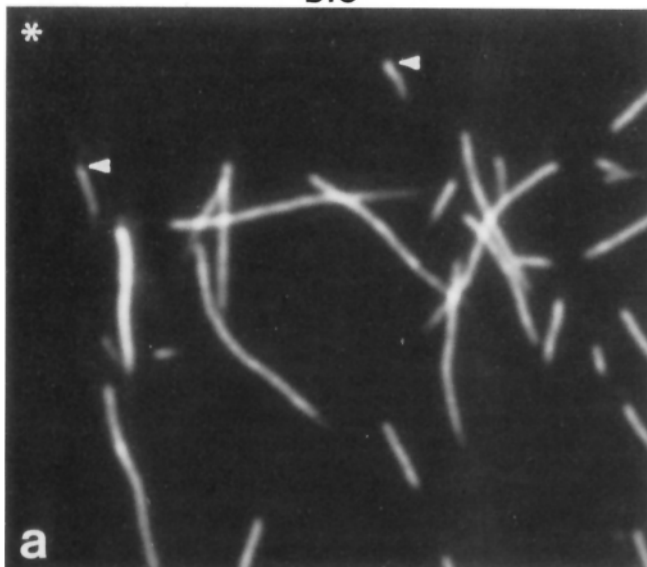
Table I. Turnover of Glu MTs Generated by TTL Inhibition

Incubation time (min)	% Glu MTs containing hapten	% Glu MTs lacking hapten	(n)
<2	77 $\pm$ 9	23 $\pm$ 9	280 13
10	90 $\pm$ 3	10 $\pm$ 3	711 11
60	100	0	501 8

Swiss 3T3 cells were incubated for 24 h in F-10 medium without serum, injected with rabbit polyclonal TTL antiserum, and incubated for another 24 h. The cells were then injected with biotinylated tubulin and incubated for the intervals specified. The cells were lysed, fixed, and processed for double-label immunofluorescence. Photographic prints of each staining pattern were traced onto clear acetate, the MT patterns were compared, and the proportions ( $\pm$ SD) in each category were tabulated. n = No. MTs/No. cells.

Bio

Glu



cells showed no detectable staining. Injected cells (Fig. 4 *f*) that were subsequently stained for Ac tubulin (Fig. 4 *g*) showed no increase in modified subunits over their uninjected counterparts, in direct contrast to the Glu staining result. However, MTs stabilized by incubating cells with 10  $\mu$ M taxol for 4 h formed tightly packed bundles that stained intensely for both Glu (Fig. 4 *d*) and Ac (Fig. 4 *h*) tubulin, showing that experimentally stabilized MTs could become acetylated. Since the injection of the TTL antibody failed to increase the level of Ac tubulin staining and injected cells retained their sensitivity to nocodazole- and  $\text{Ca}^{2+}$ -induced MT depolymerization (data not shown), we concluded that detyrosination was not sufficient to stabilize the MTs in those cells.

## Discussion

Previous studies demonstrated a correlation between stable cytoplasmic MTs and an elevated level of Glu subunits (Bré et al., 1987; Kreis, 1987; Schulze and Kirschner, 1987; Webster et al., 1987*b*). Such studies could not, however, discern whether detyrosination conferred stability on MTs or accumulated on older, stable polymer. Likewise, *in vitro* tests of the dynamics of experimentally detyrosinated MTs (Arce et al., 1978; Kumar and Flavin, 1982; Khawaja et al., 1988) were not conclusive, since cellular factors that might also have been required to retain stability might have been removed. We reasoned that the cause and effect of this association could be ascertained by specifically inhibiting the tyrosinating enzyme (TTL) *in vivo*, thereby eliciting a conservation of the entire MT population from a predominantly Tyr to a predominantly Glu array. Any significant increase in MT stability resulting from such a global transformation would presumably occur on all of the MTs in the cell and would thus be easily detected by our methods.

By each method, we found that the experimentally detyrosinated MTs of TTL antibody-injected cells were as dynamic as the Tyr MTs found in uninjected cells. Antibody-injected cells displayed no increase in Ac tubulin staining on their MTs, incorporated biotinylated tubulin first onto their Glu MT ends and then rapidly and uniformly along their entire lengths, and recovered fluorescence in bleached regions of their MTs with similarly rapid kinetics. Since staining for Tyr tubulin on MTs in these cells was absent, we concluded that these MTs were virtually all Glu and thus detyrosination was not sufficient to stabilize them. Furthermore, we concluded that, although detyrosination may be involved in maintaining the stability of particular MTs, the generation

of the stable Glu MT subset observed earlier (Webster et al., 1987*b*) must have arisen by a different mechanism.

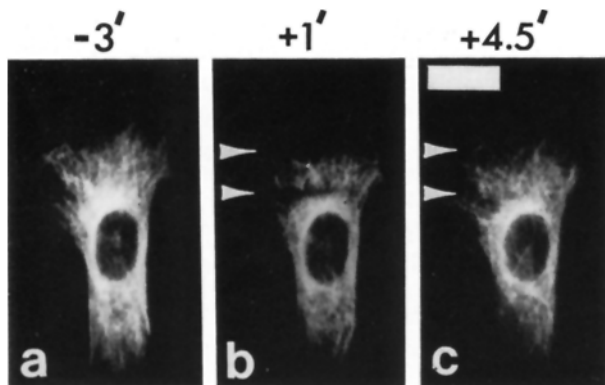
The conversion from Tyr to Glu was gradual, being first detectable  $\sim 1$  h after injection and continuing to virtual completion after  $\sim 12$  h of incubation. The first appearance of Glu immunostaining on MTs corresponded well with the reappearance of Glu MTs in 3T3 cells after their release from depolymerizing conditions (Wehland and Weber, 1987*b*) suggesting that, although TTL activity was inhibited, tubulin carboxypeptidase activity was unaffected by the antibody injection and continued at its normal rate. Thus, although TTL and tubulin carboxypeptidase activities may rise and fall in concert during specific differentiative events (Arregui and Barra, 1989; Gundersen et al., 1989), the two enzyme activities can be experimentally uncoupled.

The reappearance of Tyr staining on MTs after injection was also gradual. A partial resurgence in Tyr staining was observed  $\sim 40$ – $45$  h or two to three cell generations after injection, which continued until strong Tyr staining was again observed on all discernable MTs, suggesting that the inhibition of the TTL was waning and that the level of Tyr and Glu subunits is closely regulated. However, the interval of total TTL inhibition was long enough to examine the viability and MT turnover of Glu-enriched cells.

How do cellular MTs become stabilized? Since subunit detyrosination does not directly confer stability on MTs, individual polymers may presumably become stabilized by either capping of their (+) ends or by lateral interactions between MTs and other proteins. Although no MT capping proteins have yet been identified, their eventual identification seems likely. For example, structures that cap the plus ends of ciliary MTs have been identified and the conditions for their release have been determined (Suprenant and Dentler, 1988). Furthermore, hapten-labeled brain tubulin that is injected into fibroblasts does not assemble onto the ends of endogenous Glu MTs (Webster et al., 1987*b*) which are also resistant to drug-induced depolymerization (Bré et al., 1987; Khawaja et al., 1988), implying that their ends may be modified to prevent their disassembly. Several proteins that bind to the ends of actin filaments and inhibit their elongation have been identified (Schliwa, 1986), and it seems reasonable to predict that proteins with similar functions exist that interact specifically with MTs. Alternatively, MAPs have been shown to increase the stability of MTs *in vivo* after their microinjection into cultured cells (Drubin and Kirschner, 1986), and dampen the oscillations of MTs between growing and shrinking phases *in vitro* (Hotani and Horio, 1988), suggesting that MAPs participate in stabilizing MTs.

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*Figure 2.* Experimentally converted Glu MTs are dynamic. 3T3 cells were injected with TTL antiserum, incubated for 24 h, injected a second time with biotinylated tubulin, and further incubated for <2, 10, or 60 min before lysing and fixing the cells. Biotinylated domains (*a*, *c*, *e*) were labeled with streptavidin and Glu MTs (*b*, *d*, *f*) were immunostained as before. Most Glu MT ends in all cells incubated for <2 min after injection were capped with biotinylated domains (*arrowheads* in *a* and *b*); however, some adjacent Glu ends lacked biotin caps (*asterisk* in *a* and *b*). 10 min after injection many Glu MTs were uniformly stained for injected subunits (*arrows* in *c* and *d*), but some remained unlabeled (*asterisk* in *c* and *d*). The cell shown in *c* and *d* displays fewer biotinylated MTs than the average analyzed cell but shows more clearly the lack of full incorporation into the cytoskeleton by this time. However, 60 min after injection bulk turnover was complete and the biotin and Glu MT staining patterns were identical (*e* and *f*). Bar, 5  $\mu$ m.



**Figure 3.** Photobleached Glu MTs recover fluorescence quickly. 3T3 cells were injected first with Xrhodamine-labeled tubulin, incubated for 1–2 h, then located by rhodamine fluorescence and injected with the TTL antibody. The cells were then incubated for 12–20 h before bar-shaped regions perpendicular to the long axis of the cell were bleached with monochromatic light from an argon ion laser. The cells were monitored closely after bleaching and images were recorded periodically to document recovery. Cells injected with only Xrhodamine tubulin were also bleached for the comparison of recovery rates. The cell in *a* shows the distribution of Xrhodamine tubulin in a doubly injected cell  $\sim 3$  min before bleaching. The images in *b* and *c* show the Xrhodamine MT pattern in the same cell  $\sim 1$  min and 4 min, 43 s after bleaching, respectively. The arrowheads mark the sites of bleaching in this cell. At the latter time, the fluorescence intensity in the bleached zone had recovered to a level indistinguishable from that found in the pre-bleach image. Bar, 10  $\mu\text{m}$ .

Furthermore, fibroblasts transfected with a tau gene display thick MT bundles (Kanai et al., 1989), suggesting that these MAPs might promote MT stabilization by cross-linking them. Many stable MT assemblies (including the axonemes of cilia and flagella, the marginal bands of avian erythrocytes, and the MT core of axons) display lateral projections between MTs that may play a role in maintaining their structural integrity (Gibbons, 1981; Kim et al., 1987; Hirokawa et al., 1985). Nevertheless, rapid MT dynamics have been observed in many cell lines where MAPs exhibit a uniform distribution over all distinguishable MTs (Bulinski and Borisy, 1980; Webster et al., 1987b), implying that MAP binding alone is insufficient to stabilize particular MTs within a dense array in these cells. Although the importance of potential capping proteins and other, more completely characterized, MAPs in stabilizing MTs has not yet been established, it is now clear that the function of tubulin detyrosination is not to stabilize MTs directly, but may serve to further differentiate stable MTs for specific activities or may interact with Glu-specific MAPs to maintain stability.

The posttranslational modification of MTs may target them for specific interactions with other cell organelles or

structures. For example, it was shown that domains of acetylation occurred on stable MTs (Webster and Borisy, 1989), suggesting that this modification differentiated particular MT regions for specific functions. Similarly, detyrosination, which accumulates uniformly on MTs (Geuens et al., 1986), may mark entire MTs for functional specialization.

The *raison d'être* for tubulin posttranslational modifications remains enigmatic. The occurrence of these modifications on a limited subset of MTs in phylogenetically diverse cell types and the established correlation between tubulin modification and MT stabilization would indicate a role for them in MT function. Although no such function has yet been identified, it is important to remember that cells from most cultured lines have undergone dramatic changes in response to the artificial environment and the processes that make them immortal. Instead of proceeding through a finite number of division cycles resulting in terminal differentiation, cultured cells exist apart from their natural milieu and continue to cycle through many generations. In addition, monolayer cultures are usually studied when subconfluent, resulting in decreased interactions with neighboring cells and increased whole-cell motility. Under such conditions, the results obtained from studies of cultured cells may not determine the regulatory factors that guide MT function in cells occurring in their natural environs. Thus, although the results reported here offer an important insight into MT metabolism *in vivo*, the answers to many important questions concerning the role of posttranslational modifications in MT function must await *in situ* analyses. The posttranslational modification of MTs may be particularly important only during specific developmental stages (Rodriguez and Borisy, 1978; Gabius et al., 1983) or in response to specific extracellular signals (Nath et al., 1981), wherein significant changes in the MT array occur. Future studies that focus on the functions of these stable MTs and on the progression of developmental programs after disruption of tubulin modification cycles may provide explanations for the activity of the ubiquitously observed posttranslational modifications of tubulin.

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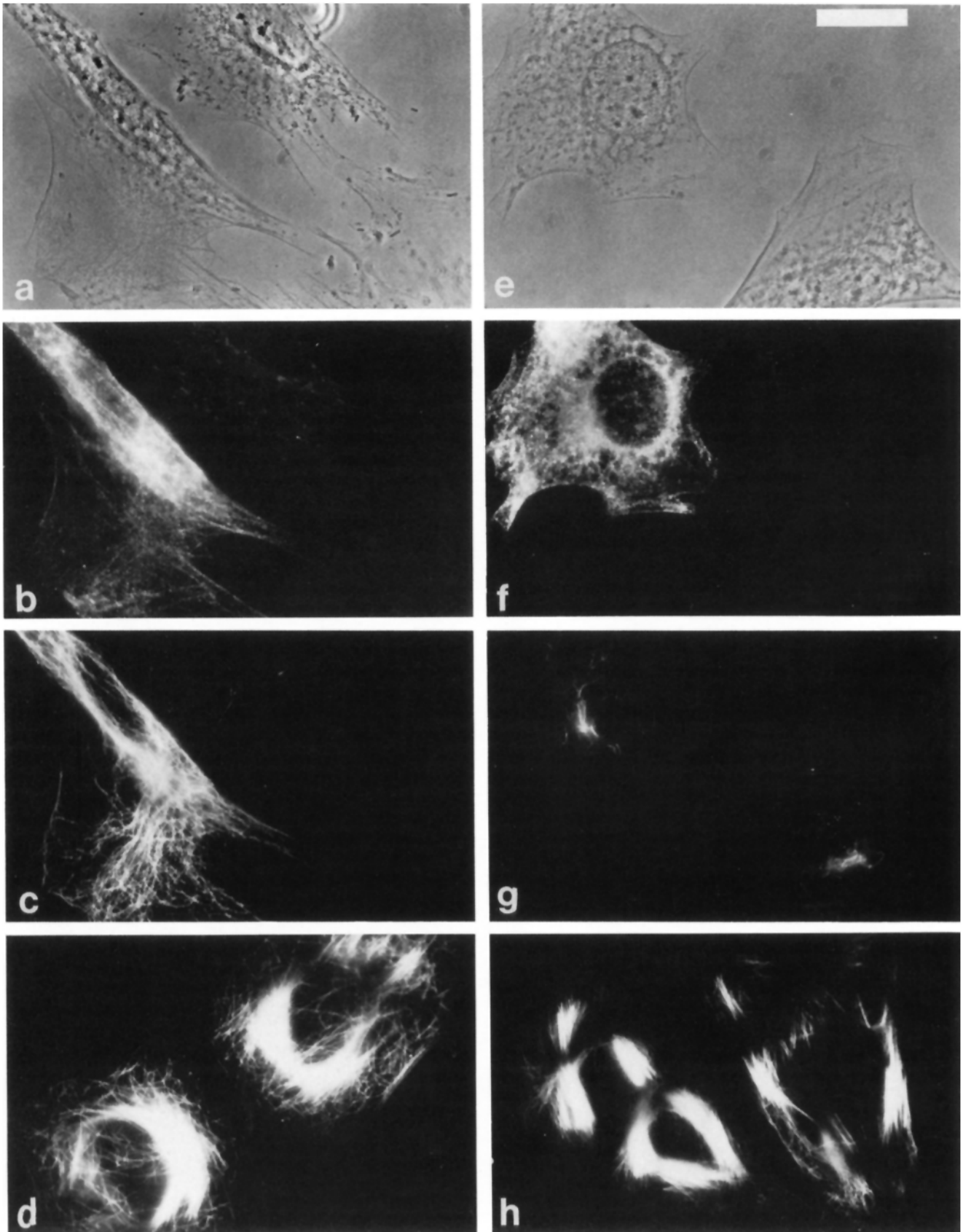
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**Figure 4.** TTL inhibition increases the level of Glu but not Ac tubulin staining in MTs. Swiss 3T3 cells were injected with the TTL antibody (the injected cells are shown on the left side of the panels) and incubated for 24 h before they were fixed in ice-cold methanol containing 5 mM EGTA. Coverslips were immunostained to detect the rabbit antibody present in injected cells (*b* and *f*) and the level of modified subunits present in all cells (Glu staining in *c* and *d*, and Ac staining in *g* and *h*). The bulk of the cytoskeleton stained brightly for Glu





subunits in TTL antibody-injected cells (c), while only a few Ac MTs were detected in other injected cells, at a level roughly equivalent to that found in untreated cells (g). However, uninjected cells treated with 10  $\mu$ M taxol for 4 h contained bundled MTs that stained intensely for both Glu (d) and Ac (h) subunits. The TTL antibody stained a few fibrous structures in (b) and (f) that resemble MTs. Bar, 20  $\mu$ m.

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