

Assembly and Turnover of Detyrosinated Tubulin in Vivo

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Abstract. Detyrosinated (Glu) tubulin was prepared from porcine brain and microinjected into human fibroblasts and Chinese hamster ovary (CHO) cells. Glu tubulin assembled onto the ends of preexisting microtubules and directly from the centrosome within minutes of its microinjection. Incorporation into the cytoskeleton continued until almost all of the microtubules were copolymers of Glu and tyrosinated (Tyr) tubulin. However, further incubation resulted in the progressive and ultimately complete loss of Glu-staining microtubules. Glu tubulin injected into nocodazole-treated cells was converted to Tyr tubulin by a putative tubulin/tyrosine ligase activity. The ob-

served decrease in staining with the Glu antibody over time was used to analyze microtubule turnover in microinjected cells. The mode of Glu disappearance was analyzed quantitatively by tabulating the number of Glu-Tyr copolymers and Tyr-only microtubules at fixed times after injection. The proportion of Glu-Tyr copolymers decreased progressively over time and no segmentally labeled microtubules were observed, indicating that microtubules turn over rapidly and individually. Our results are consistent with a closely regulated tyrosination-detyrosination cycle in living cells and suggest that microtubule turnover is mediated by dynamic instability.

How do cells regulate the assembly and disassembly of their microtubules (MTs)?¹ Many studies have demonstrated a role for the interphase centrosome, basal bodies, and the mitotic poles in nucleating the growth of MTs (20, 39, see reference 6 for review), yet it is unclear how their distribution and stability is regulated (17, 28). Variations in the complement of microtubule-associated proteins and in the tubulin dimer itself may be important factors in generating functionally distinct MT populations (7-9, 41).

Recent studies have shown that MTs rich in posttranslationally modified alpha-tubulin subunits (detyrosinated or acetylated) in cells of organisms ranging from sea urchins to humans, including such stable MT arrays as flagella and primary cilia, exist as subpopulations within the cell (13, 21) indicating that they may play an important general role in MT function (12, 29). Though the acetylation of alpha-tubulin has been correlated with flagellar assembly in *Chlamydomonas* (22-24), a functional role for alpha-tubulin detyrosination (and its subsequent retyrosination) remains obscure.

Recently, peptide-specific antibodies have been prepared which distinguish between tyrosinated (Tyr) and detyrosinated (Glu, named for the COOH-terminal glutamic acid residue of detyrosinated tubulin) alpha-tubulin (13). With these antibodies, heterogeneity in the MT population was revealed. Many MTs stained only with the Tyr antibody and

some stained only with the Glu antibody. In addition, some MTs stained with both. Cytoplasmic MTs that stained with the Glu antibody were more limited in number and length than Tyr MTs, and were characterized as having a "sinuous" or curly morphology. Electron microscopic analysis by immunogold staining of MTs confirmed the immunofluorescence results: sinuous MTs contained elevated levels of Glu subunits, while long straight MTs were enriched with Tyr subunits (10).

If the detyrosination of alpha-tubulin altered the dimer functionally, its assembly properties might be expected to differ from unmodified tubulin. The assembly properties of Glu and Tyr tubulin are similar as judged by *in vitro* assays (3, 19, 30), but correlative *in vivo* studies are lacking. We therefore investigated the assembly competence of Glu tubulin microinjected into living cells, and followed the fate of the injected Glu tubulin over time. The rapid conversion of injected Glu tubulin to Tyr tubulin suggests that the tyrosination state of alpha-tubulin is tightly controlled. In addition, our results confirm an end-mediated mode of tubulin assembly *in vivo*, and suggest a mechanism for the turnover of cytoplasmic MTs.

Materials and Methods

Preparation of Glu Tubulin

Porcine brain MT protein was stored at -70°C as a pellet after two cycles of assembly and disassembly (5). To prepare Glu tubulin, pellets were resuspended in 0.1 M K^+ Pipes, pH 6.94, 1 mM EGTA, 1 mM MgCl_2 , and 1 mM GTP to a concentration of 5-10 mg protein/ml and incubated on ice for 30 min. After removal of cold-insoluble material by centrifugation

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1. *Abbreviations used in this paper:* CPA, pancreatic carboxypeptidase A; DTAF, dichlorotriazinylaminofluorescein; Glu, detyrosinated alpha-tubulin; MT, microtubule; TTLase, tubulin/tyrosine ligase; Tyr, tyrosinated alpha-tubulin.

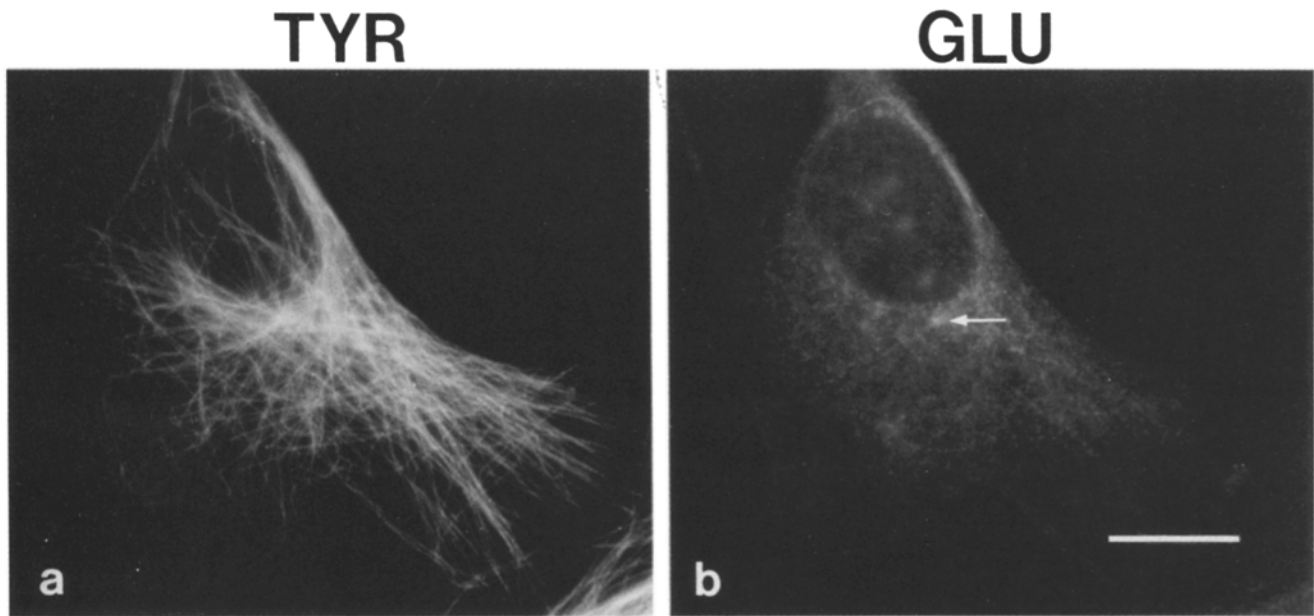


Figure 1. Untreated human fibroblasts display a typical Tyr MT pattern (a), but show no Glu-staining MTs (b). The centrosome in b stains lightly with the Glu antibody (arrow). Bar, 10 μ m.

(40,000 g for 30 min at 2°C), the supernatant was incubated with 2.5 μ g/ml of pancreatic carboxypeptidase A (CPA; Worthington Biochemicals, Malvern, PA) for 20 min at 37°C. This was the lowest concentration of CPA that completely removed the COOH-terminal tyrosine residue. After adding dithiothreitol (to 20 mM) to inactivate the CPA (19), MTs were polymerized for an additional 10 min, collected by centrifugation (40,000 g for 30 min at 35°C), and cold-depolymerized as above. The final cold supernatant was run on DEAE-Sephadex (A-50; Pharmacia Inc., Piscataway, NJ) according to Murphy (27). This preparation was cycled with 10% dimethylsulfoxide, aliquoted, and stored in liquid nitrogen. The Glu tubulin obtained by this protocol was >98% tubulin and reacted exclusively with antibodies to Glu tubulin on immunoblots probed with antibodies either to Tyr or Glu tubulin. Although the *in vitro* assembly properties of the Glu tubulin were not investigated in detail, 30–40% of the tubulin cycled through an additional round of assembly–disassembly when the DEAE fraction of brain MAPs present during the detyrosination procedure was added back to the tubulin solution. MAP degradation could account for the lower value obtained for reassembly. However, the tubulin was cycled after detyrosination to ensure that assembly-competent tubulin was injected. Dichlorotriazinylaminofluorescein (DTAF)-labeled tubulin was prepared as described earlier (39). To prepare DTAF-Glu tubulin, a frozen aliquot of DTAF tubulin was thawed and allowed to assemble in 10% dimethylsulfoxide before detyrosinating it with CPA. This material was then cycled once with dimethylsulfoxide and used immediately for microinjection.

Cells and Microinjection

Human foreskin fibroblasts (type 356) were obtained from the laboratory of Dr. R. DeMars, Department of Genetics, University of Wisconsin (Madison, WI), and cultured in Ham's F-10 medium supplemented with 15% fetal bovine serum. CHO cells were grown in F-10 medium with 10% fetal bovine serum. Both cell types were microinjected in Liebovitz's L-15 medium supplemented with 10 mM HEPES and 10–15% fetal bovine serum, having a final pH of 7.15–7.3. Microinjection procedures were as previously described (39). Aliquots of Glu tubulin (4 mg/ml) stored in liquid nitrogen were thawed and spun at 36,600 g for 30 min at 4°C before use. Cells in a preselected area of the coverslip were injected over a 4–5-min time period, and the midpoint of that time period was used as time zero. Cell viability and health after microinjection were assessed as follows: (a) cells injected with either buffer or tubulin retained a normal morphology by phase-contrast microscopy and an unaltered MT network by immunofluorescence microscopy; (b) cells injected with buffer continued to divide; (c) buffer-injected cells monitored 18 h after injection had migrated from the injection site; and (d) injected cells treated with nocodazole and then released displayed a Tyr-staining MT network indistinguishable from untreated cells.

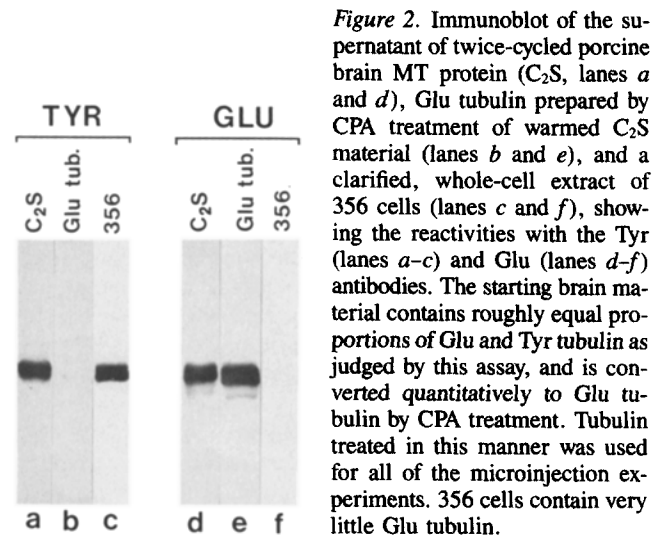


Figure 2. Immunoblot of the supernatant of twice-cycled porcine brain MT protein (C₂S, lanes a and d), Glu tubulin prepared by CPA treatment of warmed C₂S material (lanes b and e), and a clarified, whole-cell extract of 356 cells (lanes c and f), showing the reactivities with the Tyr (lanes a–c) and Glu (lanes d–f) antibodies. The starting brain material contains roughly equal proportions of Glu and Tyr tubulin as judged by this assay, and is converted quantitatively to Glu tubulin by CPA treatment. Tubulin treated in this manner was used for all of the microinjection experiments. 356 cells contain very little Glu tubulin.

Tubulin/Tyrosine Ligase (TTLase) Activity Experiments

Cells on coverslips were first treated with nocodazole to completely depolymerize all cytoplasmic MTs (25 μ M for 3 h for the human fibroblasts, 2.5 μ M for 1 h for CHO cells), and microinjected with Glu tubulin. Cells were then incubated at 37°C for established times before being released from nocodazole by three washes with drug-free medium. Cells were then incubated just long enough (5 min for CHO cells, 20 min for the human fibroblasts) to regrow MTs to the cell periphery. Cells were then fixed and prepared for double-label immunofluorescence.

Turnover Experiments

Nocodazole-treated cells were microinjected with Glu tubulin as above, then released immediately from nocodazole and incubated at 37°C for fixed times before the immunofluorescence protocol was performed. Photographic prints of staining in both the Glu and Tyr channels were then analyzed as follows. All detectable Glu MTs (40–60 per cell) were traced onto a clear acetate sheet and overlaid onto the photograph of Tyr-staining

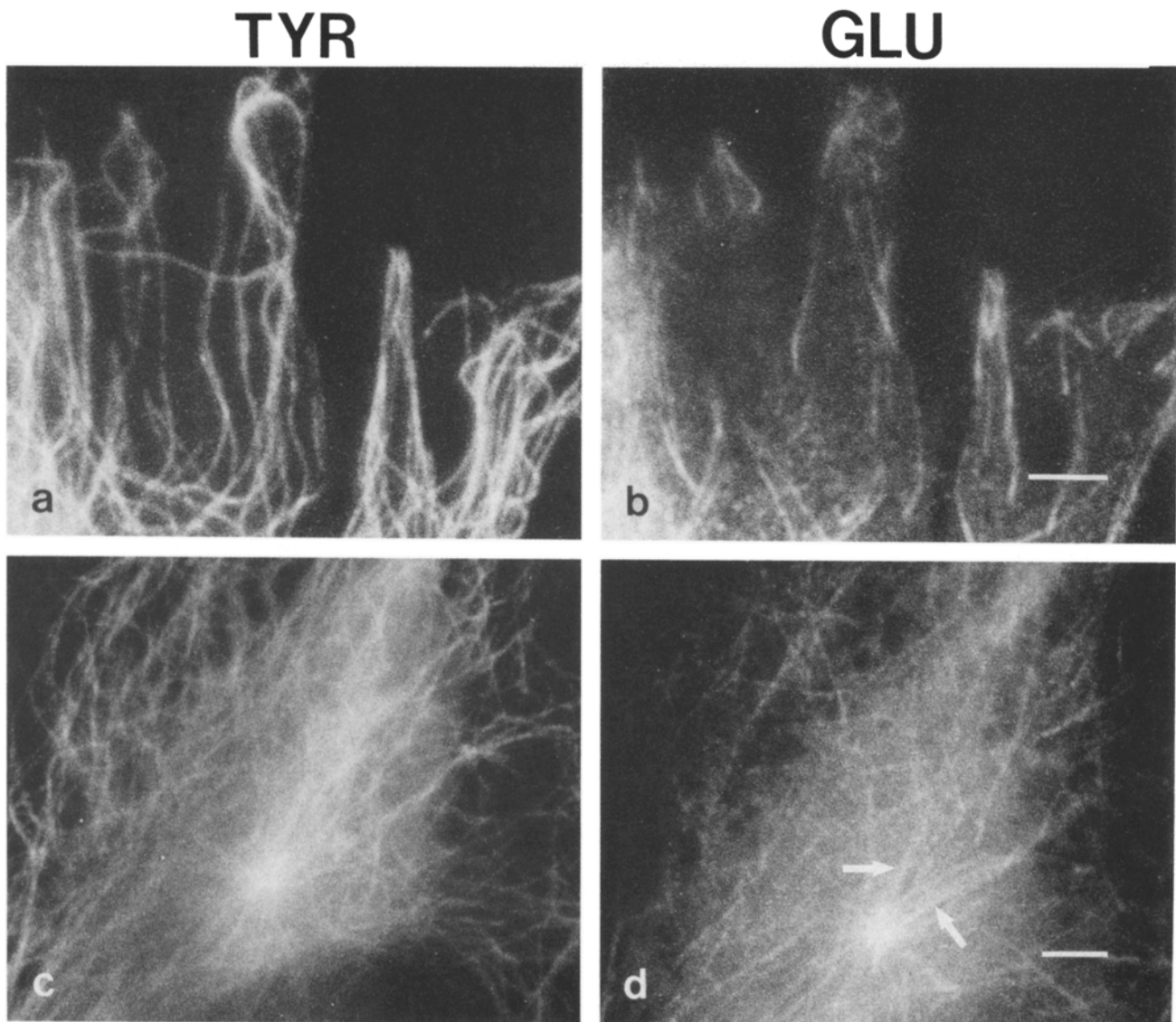


Figure 3. Glu tubulin incorporates rapidly into the cytoskeleton of 356 cells. Within 7 min of injection, Glu tubulin (*b* and *d*) assembles onto the ends of preexisting MTs (*a* and *b*) and directly from the centrosome (*arrows* in *d*). Bar, 2 μ m.

MTs from the same cell. Almost all MTs were then classified as either Glu-Tyr copolymers or Tyr-only homopolymers, based on immunofluorescence staining. A low percentage (0–8%) of MTs were classified as either Glu-only MTs or as containing Glu segments. Data from several cells per time point (150–300 total MTs counted) were routinely pooled and weighted according to the number of MTs each cell contributed to the total. For example, a cell contributing 40% of the total MTs counted for a time point would be weighted as follows: $(0.4) \times (\text{percent of MTs in classification}) / \text{total number of MTs in that time point}$. The weighted proportions were then plotted as the proportion of MTs of each classification versus time. Standard deviations were calculated weighting the value $(X - \bar{X})^2$ in the same way.

Immunofluorescence

Double-label immunofluorescence was used in all experiments. Cells grown on coverslips were rinsed briefly in PHEM buffer (36) containing 60 mM Pipes, 25 mM HEPES, pH 6.94, 10 mM EGTA, 2 mM MgCl_2 , extracted for one min in PHEM plus 0.1% vol/vol Triton X-100, and fixed with 0.7% glutaraldehyde in PHEM buffer. Unreacted glutaraldehyde was reduced with 1 mg/ml of sodium borohydride. Kilmartin's rat anti-Tyr antibody (clone YL 1/2, 1:500 dilution of ascites fluid; Accurate Chemical & Scientific Corp., Westbury, NY) was applied simultaneously with a rabbit anti-Glu antibody (1:50 dilution of serum, reference 13) or a rabbit antibody

prepared against fluorescein (1:100 dilution of serum, reference 11). Similarly, a rhodamine-conjugated anti-rabbit IgG and a fluorescein-conjugated goat anti-rat IgG (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) or fluorescein-conjugated goat anti-rabbit (Kirkegaard & Perry Laboratories, Inc.) and Texas red-conjugated goat anti-rat antibodies (Jackson ImmunoResearch, Avondale, PA) were applied simultaneously after washing the cells free of primary antibody. Coverslips were mounted in a medium containing 1 mg/ml of *p*-phenylenediamine as an anti-bleaching agent. Cells were examined with a Zeiss Universal microscope equipped with epifluorescence (Carl Zeiss, Inc., Thornwood, NY) using Planapochromat $\times 63$ (1.4 NA) and Planapochromat $\times 100$ (1.3 NA) objectives. Photographs were taken with Kodak Tri-X negative film and developed with Kodak HC-110 developer.

Results

Distribution of Tyr- and Glu-staining MTs in Untreated Mammalian Cells

Cytoplasmic MTs were colabeled with a rat monoclonal antibody specific for the Tyr form of alpha-tubulin and a rabbit

TYR

GLU

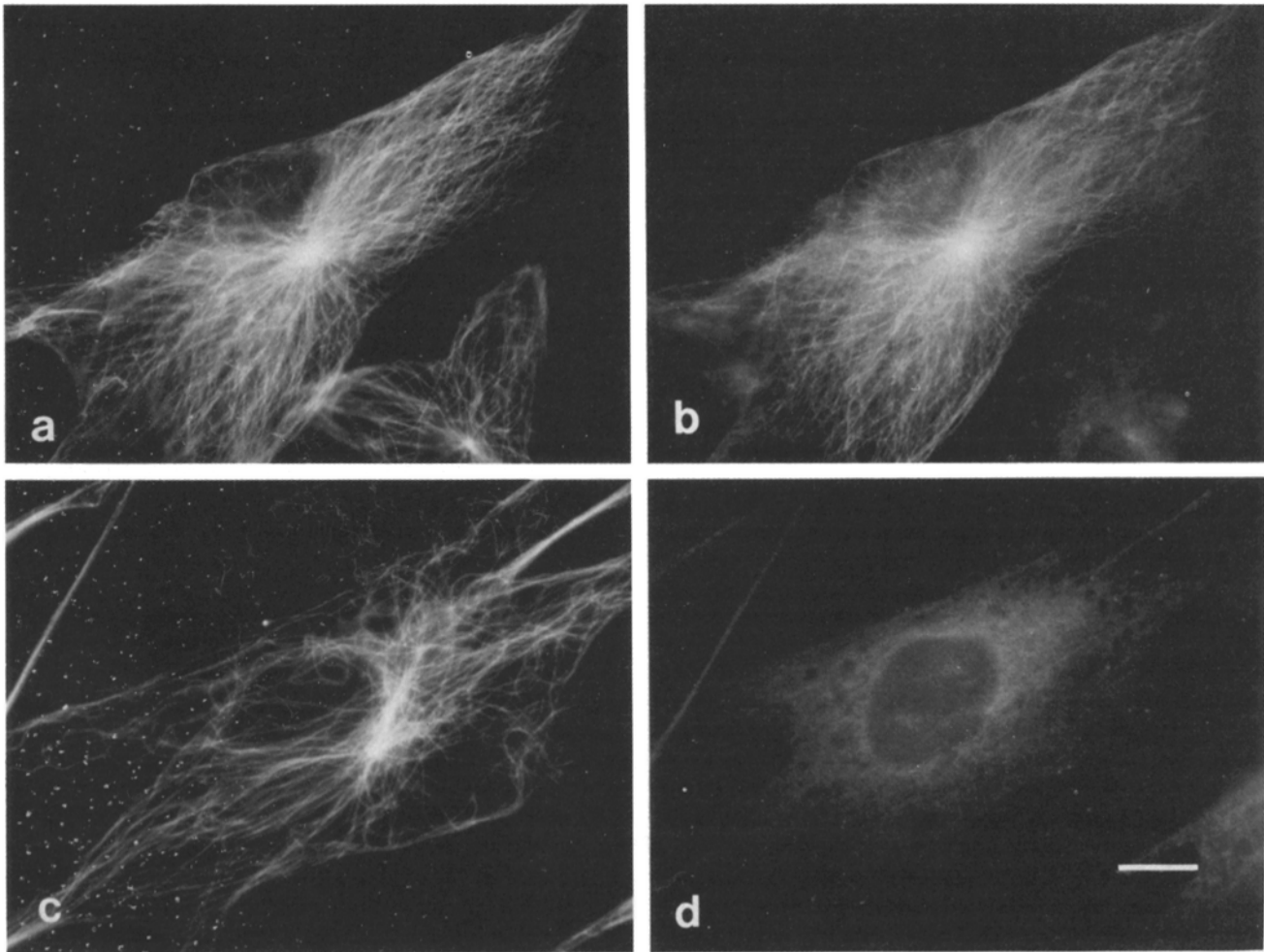


Figure 4. Injected Glu tubulin assembles uniformly into cytoplasmic MTs (*a* and *b*) of 356 cells within 15–20 min, while further incubation (90 min, *c* and *d*) results in the disappearance of Glu-staining MTs. Bar, 10 μ m.

polyclonal Glu tubulin antibody (13, 43). Tyr antibodies stained essentially the entire MT network in both cell types used (Fig. 1 *a*), and gave immunofluorescence patterns that were essentially indistinguishable from an antibody that stains both Tyr and Glu MTs (43). Glu staining of MTs was not elevated by use of increased antibody concentrations or by the omission of the Tyr antibody (data not shown). Bleed-through of Tyr staining onto the Glu-staining network was prevented by application of the secondary antibody combinations outlined in Materials and Methods.

Type 356 fibroblasts from exponentially growing cultures displayed few or no Glu MTs (Fig. 1 *b*). Occasionally, Glu MTs were observed within long cell processes ($\geq 20 \mu$ m), but these MTs often ended abruptly upon entering the cytoplasm of the cell body. In addition, short centrosomal Glu MTs were observed more frequently in confluent cultures, but still constituted a visually small subset of the total MT network. Also, the Glu antibody frequently stained the centriole as a light dot. Immunoblots of total extracts of 356 cells probed with either the Tyr or Glu antibody (Fig. 2, lanes *c* and *e*) indicated abundant levels of Tyr but low levels of Glu

tubulin, consistent with the lack of Glu staining observed by immunofluorescence. Due to the lack of endogenous Glu-staining MTs in the 356 cells and the paucity of Glu tubulin in the subunit pool, the mode of incorporation of Glu tubulin into the preexisting MT network could be examined without confusing newly labeled MTs with preexisting Glu MTs.

In contrast to the lack of Glu MTs observed in human fibroblasts, CHO cells consistently displayed Glu MTs (usually < 25 per cell), most of which arose from the centrosome. The Glu MTs were generally shorter than their Tyr counterparts, and displayed a sinuous morphology described earlier (13). MTs in CHO cells were less densely packed than in the 356 cells and did not compose parallel bundles. Thus, individual MTs were more easily identified in CHO cells and these cells were used in addition to the human fibroblasts to quantify the distribution of Glu and Tyr MTs after microinjection of Glu tubulin.

Incorporation of Glu Tubulin into Cytoplasmic MTs

Porcine brain MTs were detyrosinated with CPA (Fig. 2, lanes *b* and *e*), and subsequently the purified Glu tubulin was

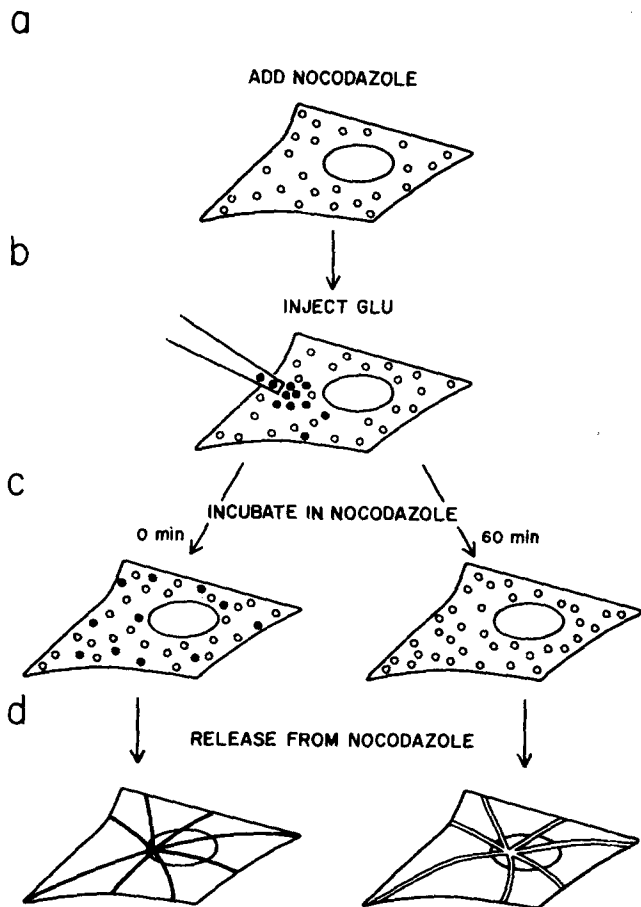


Figure 5. Schematic drawing of the assay used to detect the activity of tubulin/tyrosine ligase. Cells were first treated with nocodazole (a) to depolymerize cytoplasmic MTs. After injection of Glu tubulin (b), cells were incubated for either short or longer periods of time (c) before release from nocodazole (d). MTs from injected cells were then analyzed by immunofluorescence for the presence of Glu MTs. (Open circles) Tyr subunits; (solid circles) Glu subunits.

microinjected into human fibroblast cells. We microinjected $\sim 5\%$ of the cell volume with 4 mg/ml tubulin, an amount which has been estimated to increase the dimer pool by $\sim 15\%$ (39). Glu staining was observed at the distal (+) ends of preexisting MTs at the cell periphery within 7 min of Glu tubulin microinjection (Fig. 3, a and b). Glu-labeled segments were also observed closer to the centrosome. However, due to a greater congestion of MTs in these regions, it was not determined whether these Glu segments labeled the ends of centrosomal MTs or represented free MTs. With few exceptions, the peripheral Glu-staining MTs always stained with the Tyr antibody as well. In addition to end-labeling, short MTs arising directly from the centrosome stained with the Glu antibody, suggesting that new MT growth occurred during this time (Fig. 3, c and d). Within several minutes of injection, CHO cells had already incorporated Glu tubulin uniformly into many MTs, and end-labeling was no longer apparent.

With longer incubation, the injected 356 cells displayed continuous Glu staining along most of their MTs. The cell in Fig. 4 (a and b) was fixed 15 min after microinjection, and shows a network of Glu MTs that is very similar to the Tyr

pattern. End-labeled MTs were no longer observed and Tyr-only MTs were found infrequently, suggesting that the injected Glu tubulin had, by this time, become uniformly incorporated into MTs, and that the cytoplasmic network had turned over within that time. In a typical experiment, from 20 to 30 cells per coverslip were injected over a period of 4–5 min, and minor heterogeneity in the staining pattern was observed. For example, the intensity of Glu staining varied slightly in an average of one or two cells per coverslip, depending on the actual volume of material injected and whether a particular cell was injected first or last. In general, the pattern of staining was very consistent, and by 15–20 min the maximum number of Glu-staining MTs was observed.

Retyrosination of Injected Glu Tubulin when MTs Are Absent

Glu staining of MTs from microinjected cells disappeared with time (Fig. 4, c and d). After 90 min of incubation, Glu MTs were detected in only a very few injected cells. The most likely explanation is that the injected Glu tubulin had been retyrosinated, presumably by the endogenous TTLase. In vitro studies have shown that the tubulin dimer is the preferred substrate for retyrosination (3, 30). Therefore, putative TTLase activity was assayed by first depolymerizing cytoplasmic MTs with nocodazole to provide the optimal substrate for the enzyme. Selected cells were then microinjected with Glu tubulin and incubated for increasing times in the presence of nocodazole before the MTs were allowed to regrow in the absence of drug (Fig. 5). As shown in Fig. 6, a and b, human fibroblasts injected with Glu tubulin and incubated in nocodazole-containing medium for 30 min before drug release still contained enough Glu tubulin to be observed as Glu MTs by immunofluorescence. However, cells incubated for 60 min and then released from nocodazole showed no Glu staining, indicating that the amount of Glu tubulin remaining was significantly less than at the earlier time point (Fig. 6, c and d) and had fallen below the threshold of detection. Cells released from nocodazole after less than 30 min showed qualitatively similar Glu staining patterns, though possibly greater in intensity. 30 min was the longest incubation time in nocodazole after which, following drug release and MT regrowth, Glu staining of MTs was consistently observed. Similar results were obtained using CHO cells.

An alternative explanation for the loss of Glu staining over time would be that the injected Glu tubulin gradually lost its ability to assemble into MTs. To test the continued assembly competence of injected Glu tubulin independently of its retyrosination, tubulin labeled with DTAF was detyrosinated and injected into nocodazole-treated cells as before. The fluorescein hapten marked injected subunits, while the detyrosinated tubulin provided a substrate for the TTLase. Injected cells were then either released immediately from the drug or after an additional 60-min incubation in nocodazole, and stained with either the fluorescein antibody or the Glu antibody and counterstained with the Kilmartin antibody (Fig. 7). Cells released from nocodazole immediately after injection showed intense Glu-staining MTs and weaker labeling with the anti-fluorescein antibody (Fig. 7, a and c). In both cases, injected cells displayed a normal Tyr-staining MT array (Fig. 7, b and d).

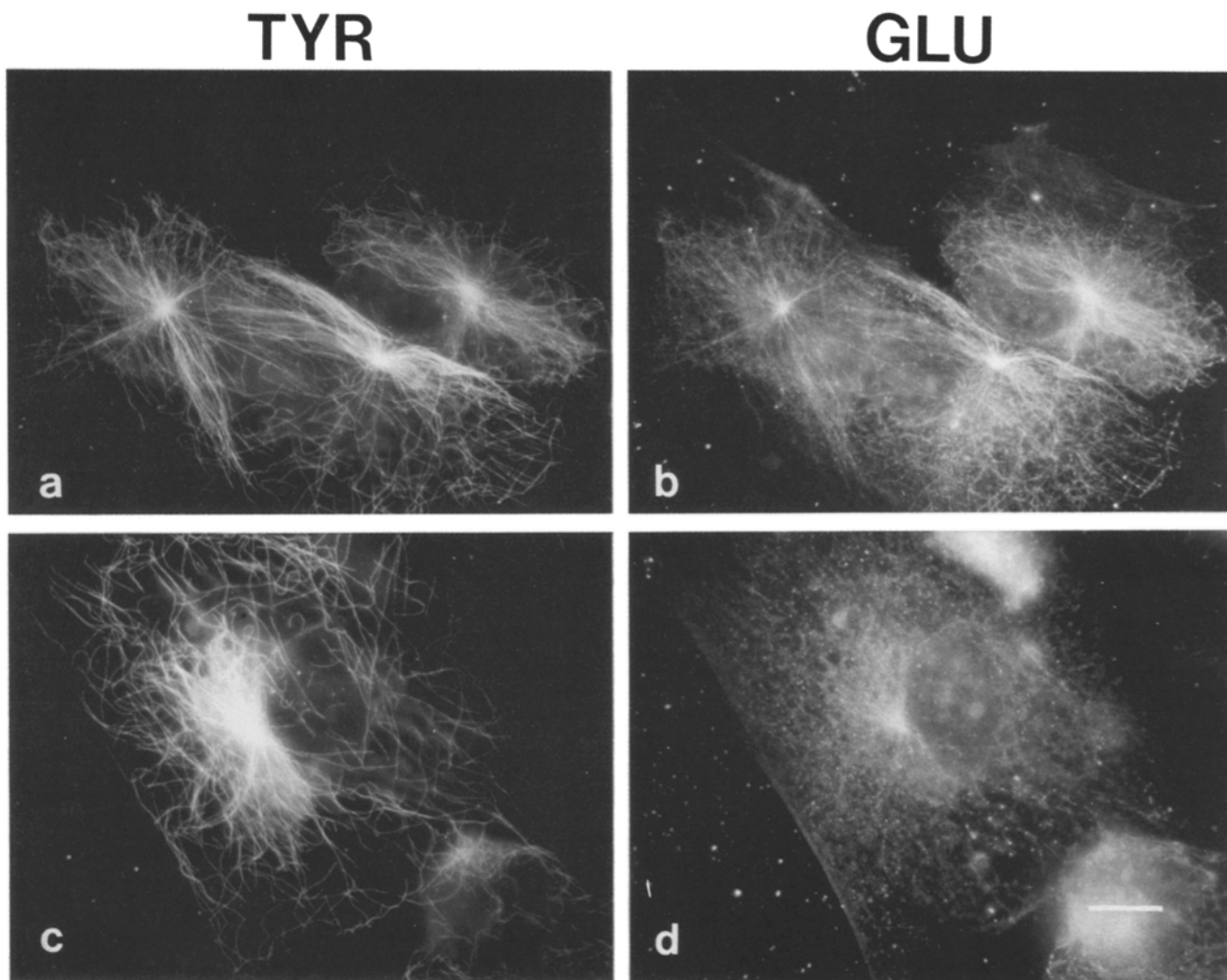


Figure 6. Typical results from an assay for tubulin/tyrosine ligase activity on 356 cells. Injected cells incubated in nocodazole for 30 min before drug release display MTs composed of both Glu and Tyr subunits (*a* and *b*). Cells incubated for 60 min before release show only Tyr-staining MTs (*c* and *d*). Bar, 10 μ m.

Cells released from nocodazole 60 min after injection of DTAF-Glu tubulin showed staining for the fluorescein hapten similar in intensity to that observed immediately after injection (Fig. 7 *e*). At this time Glu staining in similar cells was absent (Fig. 7 *g*), though Tyr-staining MTs remained (Fig. 7, *f* and *h*). By this criterion, injected Glu tubulin remained able to assemble into MTs at the time that staining for the Glu subunits disappeared, indicating that the loss of Glu staining occurred via retyrosination of the injected dimers.

Retyrosination of Injected Glu Tubulin when MTs Are Present

The turnover of tyrosine on MTs was studied in more detail. As depicted in the flow diagram in Fig. 8, nocodazole-treated 356 and CHO cells were injected with Glu tubulin and released immediately from the drug, allowing immediate and uniform Glu tubulin incorporation into the cytoskeleton. At specific times after release injected cells were fixed and prepared for immunofluorescence. Injected 356 cells allowed to recover only a short time displayed asters of MTs composed

of both Glu and Tyr subunits (Fig. 9, *a* and *b*). With longer incubation, Glu staining was uniform over a majority of the cytoplasmic MTs and later disappeared (Fig. 9, *c-f*), in a manner similar to that depicted in Fig. 4.

For each time point after release, the proportion of cytoplasmic MTs that stained with the Glu antibody was tabulated by counting the number of Glu MTs visible in immunofluorescence micrographs and comparing that number with the number of Tyr-staining MTs from the same cell. A Glu or Tyr MT is defined here as any MT that stains visibly by immunofluorescence with the appropriate antibody, and may not reflect the absolute amount of each species present in each MT (10). Glu MTs that did not also stain with Kilmartin antibody were rare. Fig. 10 shows that Tyr MTs can be discerned from Glu-Tyr copolymers by comparison on photographic prints of the staining patterns obtained with the two antibodies. It should be noted that, as represented by the MTs seen here, staining heterogeneity is rarely observed; MTs are usually either Tyr homopolymers or Glu-Tyr copolymers along their observable lengths. After classifying all discern-

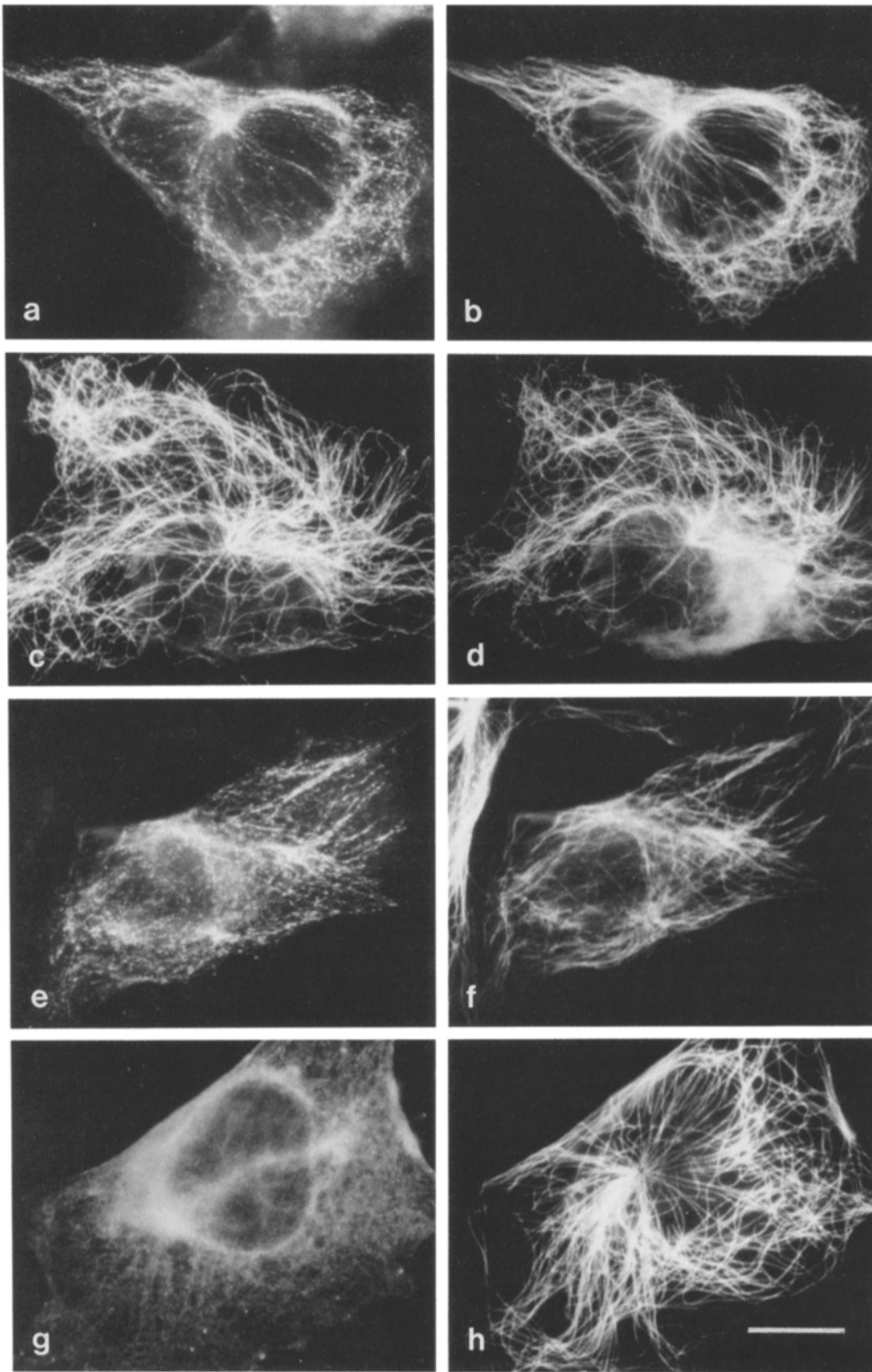


Figure 7. DTAF-Glu tubulin injected into 356 cells retains its assembly competence but loses its reactivity with the Glu antibody. Cells were double-labeled with either anti-fluorescein and Kilmartin antibodies, or Glu and Kilmartin. Nocodazole-treated cells released from the drug immediately after injection (*a-d*) display DTAF labeling (*a*) that mimics the Tyr-staining pattern (*b*), while the intensity of Glu staining in identically treated cells (*c*) is high and also resembles the Tyr-staining network (*d*). Cells released from nocodazole 60 min after injection (*e-h*) show a DTAF pattern similar in intensity to that observed in *a*, while Glu staining of an identically treated cell is absent (*g*). Tyr staining is displayed in *b*, *d*, *f*, and *h*. Bar, 10 μ m.

able MTs as either Tyr homopolymers or Glu-Tyr copolymers (or rarely, segmentally labeled MTs), the number of each type was converted to the proportion of the total MT population and plotted against time.

In each individual 356 cell the proportion of Glu-Tyr copolymers decreased progressively over time (Fig. 11, left), corresponding to a complementary increase in Tyr-only MTs,

which suggests that the transition from Glu to Tyr occurred MT by MT and not to the general MT population at once. At early times <100% of the MTs were counted as Glu, and this might be accounted for by the poorer resolution of dimer Glu MTs within the asters, though some type of subunit sorting mechanism cannot be ruled out. Immunofluorescence staining of *in vitro* polymerized brain tubulin with Glu and

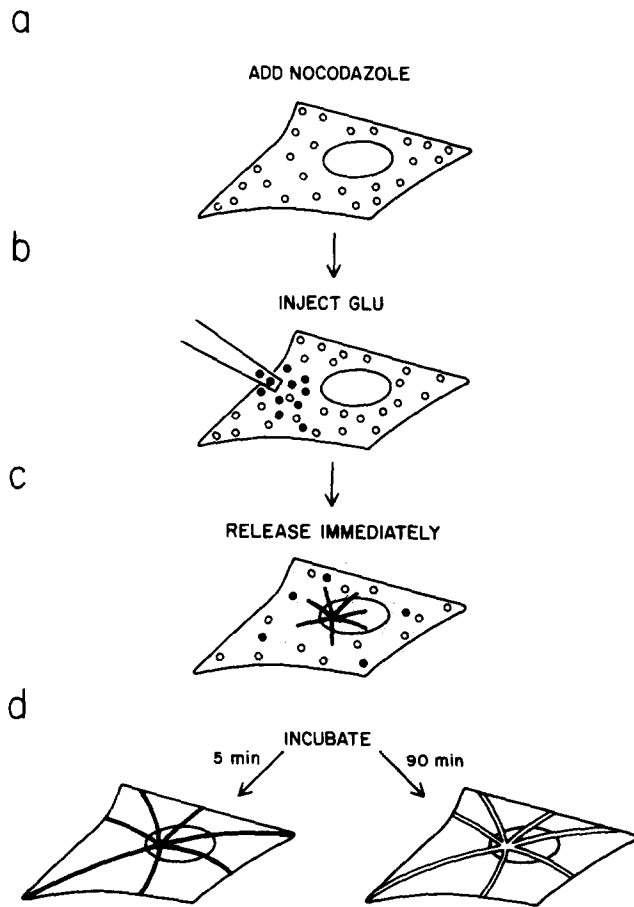


Figure 8. Schematic drawing of the assay used to analyze turnover of Glu tubulin in MTs. MTs were depolymerized with nocodazole as in the ligase assay (a), but after injection of Glu tubulin (b) cells were released immediately from the drug (c), and incubated for short or long periods of time before fixing (d). (Open circles) Tyr subunits; (solid circles) Glu subunits.

Tyr antibodies has failed to reveal any such sorting (our unpublished observation). By 90 min the proportions of Glu-Tyr copolymers and Tyr-only homopolymers approached the levels found in uninjected cells. A similar pattern of loss of Glu fluorescence was observed when the nocodazole treatment was omitted, but the time of maximum Glu tubulin incorporation (time zero in the turnover experiments) was then more difficult to determine consistently.

Turnover of tyrosine in the presence of MTs was also investigated in CHO cells for two reasons. First, the radially oriented MT array facilitated the detection and classification of individual MTs. Second, it was of interest to analyze tubulin tyrosine turnover in a cell type that contained appreciable numbers of Glu MTs. As for 356 cells, immediately after release from nocodazole, Glu tubulin appeared in almost all MTs. However, in contrast to 356 cells, CHO cells still displayed Glu MTs at long times after injection. In no instance was a progressive shortening of Glu MTs observed. As seen in Fig. 11 (right), the progressive loss of Glu-staining MTs was accompanied by an increase in the proportion of Tyr-only MTs until approximate steady-state values were attained, as in the results obtained for 356 cells.

Discussion

In an attempt to understand the function of Glu MTs within the cell, we first assessed the ability of Glu tubulin, a naturally occurring, posttranslationally modified form of tubulin, to assemble into cellular MTs. In an earlier study (39), fluorescein-haptenized tubulin was found to incorporate into cellular MTs by an end-mediated process. Having used a different probe (Glu tubulin) in the same cell type (human fibroblasts), we now confirm the earlier results that tubulin assembles rapidly at the distal ends of preexisting MTs and directly from the centrosome. These results are also consistent with the *in vitro* assembly properties of Glu tubulin, which are essentially indistinguishable from those of Tyr tubulin (3, 19, 30). With further incubation Glu tubulin was found to incorporate uniformly into the MT network, as in the Tyr MT pattern, during a time course similar to that found in other cells injected with tubulin derivatized with fluorescein (39) or biotin (38) haptens.

Unlike the results found with DTAF or biotinylated tubulin, Glu staining of microinjected cells disappeared with time. We considered two possibilities: (a) the microinjected Glu tubulin incorporated into MTs, reentered the cytoplasmic pool, and eventually became assembly incompetent through some unspecified mechanism, or (b) the microinjected Glu tubulin became retyrosinated by an endogenous TTLase activity. The results of the ligase assays where DTAF-labeled Glu tubulin was injected demonstrated the continued ability of the injected subunits to assemble into MTs. Thus, it was concluded that the observed loss of Glu fluorescence arose not from the limited half-life of the injected tubulin but from its modification (tyrosination) after injection.

Injected Glu tubulin was rapidly converted to Tyr tubulin. In nocodazole-treated 356 cells, between 30 and 60 min was required to drop the level of Glu subunits below the threshold of visibility of immunofluorescence. Likewise, a similar amount of time was required from the point of maximum incorporation of Glu tubulin into MTs (15–30 min) to its disappearance in otherwise untreated cells (90 min, Fig. 4). Could an endogenous TTLase account for this conversion of injected Glu subunits to Tyr? From the estimated number of ligase molecules per cell (4×10^3 , references 31 and 37) and an estimated turnover number of 30 tyrosines per ligase molecule per minute (37), an average tissue culture cell could tyrosinate roughly 3.6×10^6 dimers within 30 min, which exceeds the estimated 3×10^6 Glu dimers injected (15, 39). Given the approximate nature of the assumptions, we consider the agreement to be good and we conclude that the endogenous TTLase could convert all of the injected Glu tubulin within the time span of a routine ligase assay. Similar rates of tyrosine turnover were obtained by Thompson et al. (40) in cultured muscle cells, where they found the half-life of tyrosine on alpha-tubulin at steady-state to be ~ 37 min.

The observed loss of Glu-staining MTs over time provided us with a tool with which to investigate MT turnover, but its use required that two assumptions be met. First, we assumed that TTLase activity occurred predominately on free tubulin subunits. *In vitro* studies (3, 30) support this assumption. In addition, the TTLase needed to work rapidly enough for loss of Glu fluorescence on MTs to be correlated with MT turn-

TYR

GLU

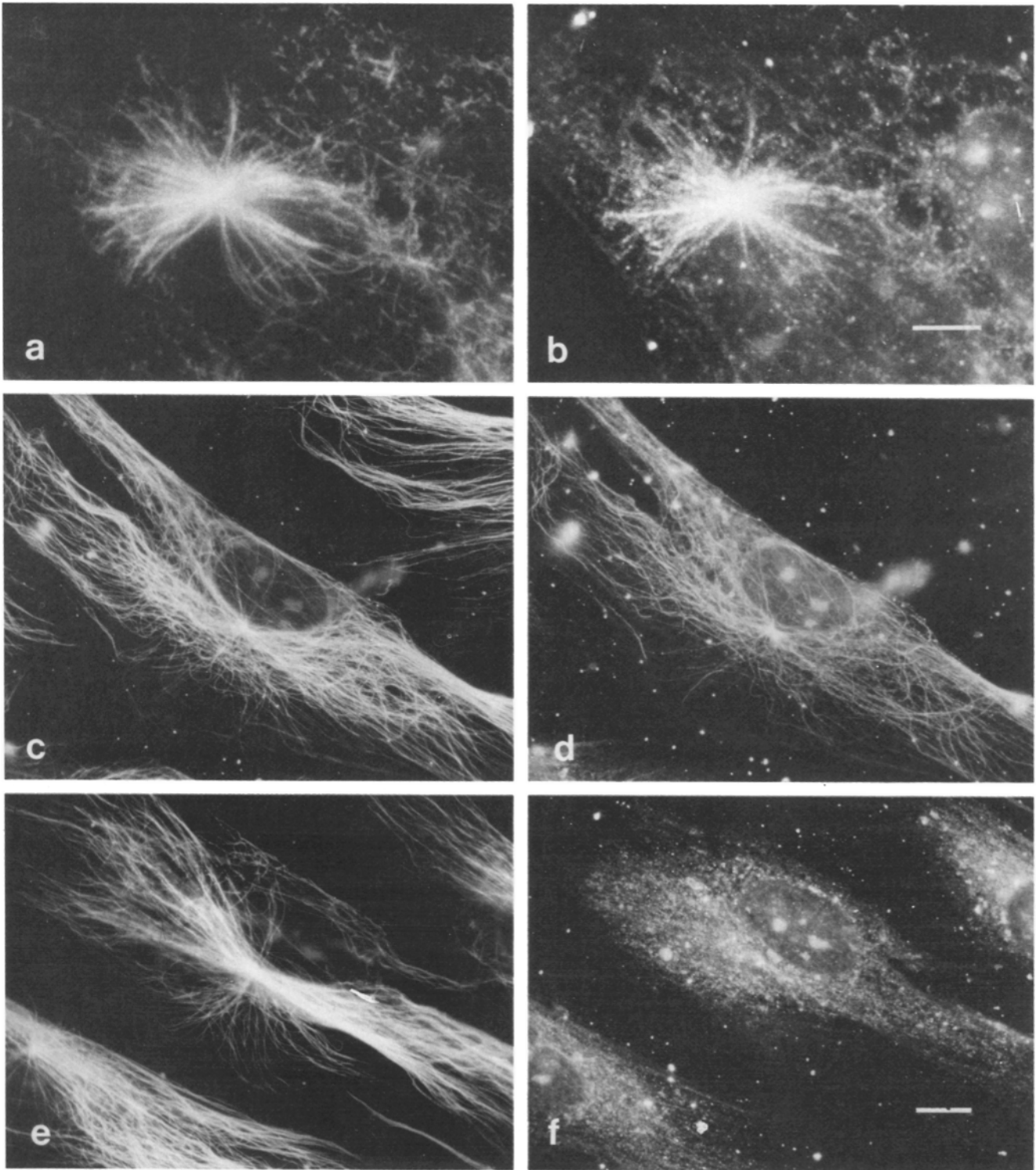


Figure 9. 356 cells released from nocodazole immediately after Glu tubulin injection and incubated for 9 min regrow asters which are copolymers of Glu and Tyr tubulin (*a* and *b*). After a 30-min incubation there are still many Glu-staining MTs present (*c* and *d*), but after 90 min of incubation only Tyr-staining MTs remain (*e* and *f*). Bars: (*a* and *b*) 5 μ m; (*c-f*) 10 μ m.

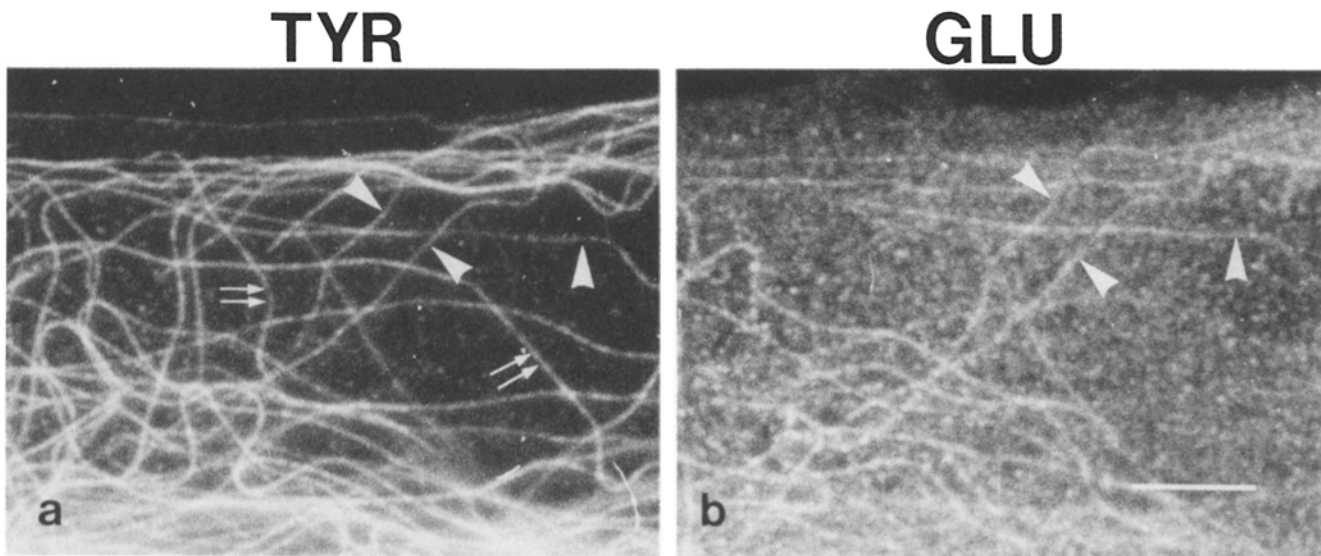


Figure 10. High magnification micrograph of an area of a 356 cell incubated for 30 min after Glu tubulin injection, displaying both MTs that stain only with a Tyr antibody (*double arrows*) and Glu-Tyr copolymers (*large arrowheads*). There is no staining heterogeneity along the length of these MTs. MTs of both classes were tabulated and plotted for each time point assayed (Fig. 11). Bar, 5 μ m.

over. The similarity in the kinetics of retyrosination between cells treated with or without nocodazole confirmed that Glu subunits released from MTs would be quickly retyrosinated, most likely before readdition onto a different MT.

Haptenized tubulins have allowed the kinetics of incorporation into MTs to be examined, but the conclusions have necessarily been qualified due to perturbation of the tubulin pool size caused by the injection and the possible modification of the assembly kinetics due to the derivatized tubulin. In this study injected cells were allowed to incorporate Glu tubulin uniformly into cytoplasmic MTs before the analysis of MT turnover was made, minimizing the problem of injection perturbation. It is conceivable that the elevated level of Glu subunits within MTs during the assay perturbed normal

dynamics. However, the rate of incorporation of Glu dimers onto preexisting MTs was similar to that observed with haptenized tubulin probes.

The turnover of COOH-terminal tyrosine on alpha-tubulin was considered in light of three current models for MT dynamics: direct polymer modification, treadmilling, and dynamic instability (Fig. 12). In the polymer modification model retyrosination would occur either directly on the polymer or via subunit exchange along the entire length of MTs. This model would predict a gradual and uniform dimming of Glu immunostaining fluorescence on all MTs until the threshold of detection was reached. This gradual and uniform dimming was not observed. This negative result is consistent with the demonstrated preference of TTLase for tubu-

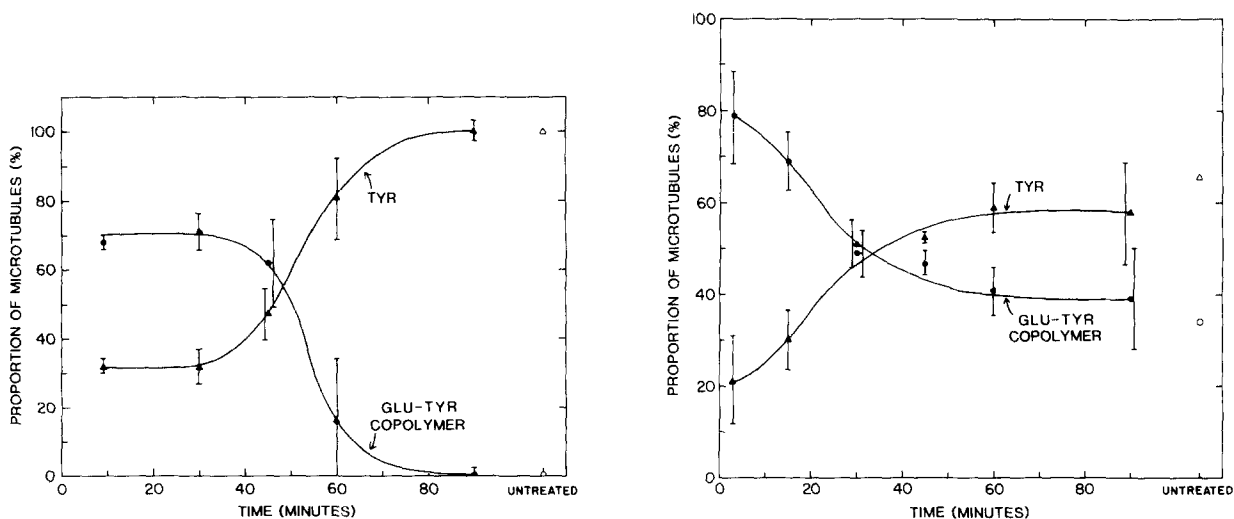


Figure 11. Turnover of Glu MTs in human fibroblasts (*left*) and CHO cells (*right*). Nocodazole-treated cells were injected with Glu tubulin and then immediately released from the drug block to permit the formation of Glu-Tyr copolymers. With time after reformation of the MT array, the proportion of Glu-Tyr copolymers (*solid circles*) decreased progressively while the proportion of Tyr MTs (*solid triangles*) increased in a complementary manner until steady-state levels were reached (*open symbols*).

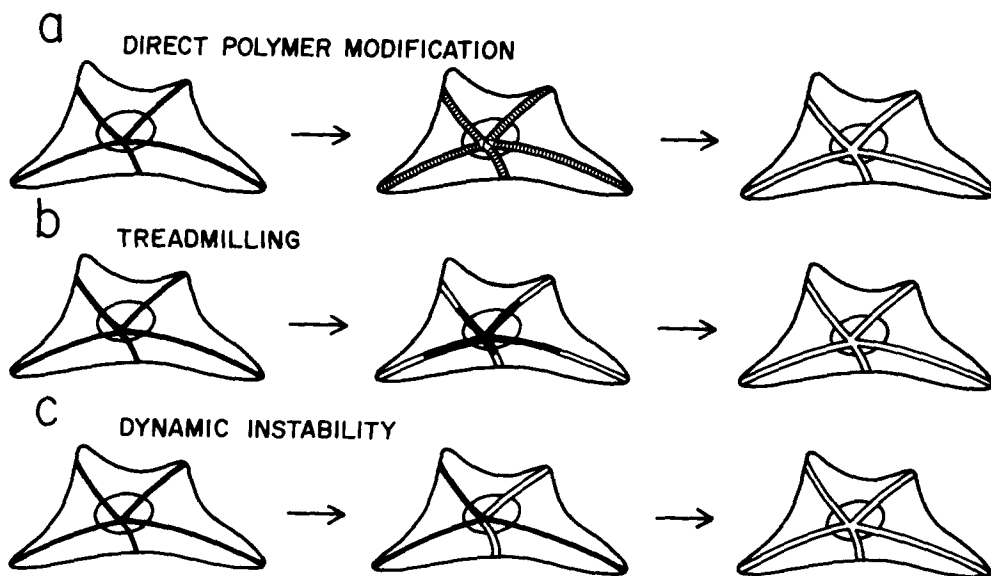


Figure 12. Three possible models for the turnover of the COOH-terminal tyrosine of alpha-tubulin. In *a* tyrosine is either added directly to the polymer-bound alpha subunit, or detyrosinated subunits exchange with the soluble pool along the entire length of the MT. In *b* tyrosine is added to free dimers that have entered the subunit pool by MT treadmilling, and in *c* retyrosination occurs on free dimers released from the polymer pool via dynamic instability.

lin dimers (3, 30) and tubulin/tyrosine carboxypeptidase (the specific detyrosinating enzyme) for MTs (1, 2, 14, 18), and does not support direct polymer modification as the mode of MT tyrosination.

In the treadmilling and dynamic instability models, loss of Glu-Tyr copolymer would occur by end-wise exchange of MT subunits with the subunit pool. Treadmilling would be detected as a gradual shortening of Glu MT domains proximal to the centrosome accompanied by a lengthening, at the same rate, of Tyr domains at the distal ends of the MTs. Treadmilling would predict a high level of segmentally labeled MTs during turnover, especially at time points after injection corresponding to virtually complete dimer retyrosination by the TTLase (30–60 min in a ligase assay). At these times, Glu-Tyr copolymers were observed side by side with Tyr MTs, and did not exhibit any observable staining heterogeneity along their lengths. Previous studies have indicated the *in vitro* rate of MT treadmilling to be on the order of micrometers per hour (4, 25, 32). If similar rates occurred *in vivo*, TTLase activity should reduce the level of Glu dimers sufficiently for segmental labeling to be observed at intermediate stages of MT turnover. Further, treadmilling would be much too slow a mechanism to account for the turnover rates recorded in this study. In accordance with this view, recent photobleaching studies (11, 33, 42) have failed to observe treadmilling in the central spindle of various cells during mitosis. Though MT dynamics might be fundamentally different in mitosis, they are also reportedly more rapid. In addition, treadmilling has not been observed in studies of interphase cells (34, 35). It therefore seems unlikely that treadmilling could account for the loss of Glu fluorescence observed in this study.

The dynamic instability model, also derived from *in vitro* studies (16, 26), suggests that MTs exchange subunits with the dimer pool by depolymerization of individual MTs after the loss of their GTP cap. The loss of Glu fluorescence from individual fibers over time can be explained by this model, since the Glu subunits released from an MT would become available to the TTLase for retyrosination before reassembly into a new MT. The model would predict that individual MTs

would lose their Glu subunits as they depolymerized and would then regrow using newly tyrosinated subunits. Since depolymerization of MTs occurs individually, dynamic instability would predict that Glu-Tyr copolymers coexist side by side with Tyr MTs at intermediate stages of the turnover process, as was in fact observed. This hypothesis is also compatible with the observed level of TTLase activity in both cell types studied. The rate of conversion of Glu to Tyr tubulin in 356 cells is faster than in CHO cells (Fig. 11). Whether this is due to more uniform MT turnover or to other factors, such as the balance between endogenous TTLase and carboxypeptidase activities, is not known.

In conclusion, the observed progressive loss of Glu MTs with time strongly suggests that MTs turn over rapidly and individually as predicted by dynamic instability. The results are compatible with a cyclic model for the tyrosination-detyrosination of alpha-tubulin (14). Although Glu tubulin is assembly competent, under steady-state conditions in cells most unpolymerized tubulin would be in the Tyr form because of an active TTLase. Detyrosination would occur principally as a postpolymerization process through a tubulin carboxypeptidase bound to the MTs. Subsequent depolymerization would release Glu subunits for retyrosination. The function of the tyrosination-detyrosination cycle and its relation to the turnover of MTs as mediated by dynamic instability are important questions for further study.

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