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Differential turnover of tyrosinated and detyrosinated microtubules

(microtubule dynamics/tyrosination/triple-label immunofluorescence/microinjection)

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ABSTRACT **Turnover of tyrosinated and detyrosinated microtubules ([Tyr]MTs and [Glu]MTs, respectively) was analyzed by the combined use of hapten-mediated immunocytochemistry and peptide-specific antibodies. Cells were microinjected with hapten-labeled tubulin and then processed for triple-label immunofluorescence to determine the pattern of incorporation of the injected subunits into [Tyr]- and [Glu]-MTs. Within 2 min of microinjection, hapten-labeled domains were present at the ends of virtually all [Tyr]MTs but were absent from most [Glu]MTs, demonstrating that [Tyr]MTs grew, whereas most [Glu]MTs did not. After 1 hr of incubation, all [Tyr]MTs analyzed were copolymers of endogenous and hapten-labeled subunits, indicating complete and rapid turnover of these MTs. However, the majority of [Glu]MTs were not hapten-labeled, indicating that they had not turned over. Even 16 hr after injection, cells that had not divided retained a small proportion of [Glu]MTs lacking hapten, implying that some had persisted for most of a cell generation. At mitosis, all MTs were hapten-labeled, indicating that the stable interphase [Glu]MTs had depolymerized. The results establish that the MT network is heterogeneous in its turnover rate, being composed of at least two populations: [Tyr]MTs that turn over rapidly and [Glu]MTs that turn over slowly.**

Implicit in the concept of the cytoskeleton is the relative permanence of the fiber systems that comprise it. It came as a surprise, therefore, that microtubule (MT) populations in proliferating cells turn over rapidly, exchanging subunits with the soluble pool with a half-time of 5–20 min (1–5). This implies that in a typical cultured cell with a generation time of 22 hr, the entire MT network turns over ≈20 times during a single cell cycle.

However, some processes such as mitosis and cell morphogenesis apparently require longer-lived MTs (6–8), and some mechanism(s) of selective stabilization, therefore, must exist. Differential incorporation of genetically distinct tubulin isotypes had been thought a possibility (9–12), but it has been shown (13) that they assemble indistinguishably into cytoplasmic and mitotic MTs. Other possible mechanisms include differential interaction with microtubule-associated proteins (14–16) and post-translational modification with phosphate (17), acetate (18, 19), or tyrosine (20, 21).

Tyrosine is cyclically added to the carboxyl terminus of unassembled α-tubulin subunits by tubulin/tyrosine ligase (21, 22) and removed from assembled subunits by a tubulin-specific carboxypeptidase leaving a glutamic acid as the carboxyl-terminal residue (23). The correlation between these enzyme activities and the tubulin assembly states suggests a role for tubulin tyrosination in regulating MT dynamics.

It has been established that cells contain subsets of MTs distinguished by whether the α-tubulin subunit is tyrosinated ([Tyr]tubulin) or detyrosinated ([Glu]tubulin) (24, 25), termed [Tyr]MTs and [Glu]MTs, respectively, and that [Glu]tubulin is enriched in certain stable MT structures (26). Not all MTs within a cell turn over with the same kinetics (27, 28) or are equally labile to depolymerizing conditions (28–31). Since the majority of MTs in many tissue culture cell types contain predominantly [Tyr]tubulin subunits (5, 24, 25, 32), it seemed likely that the turnover half-times determined earlier reflected the characteristic dynamics of the [Tyr]MT population and may have obscured the behavior of [Glu]MTs.

In this study we examined the turnover of [Tyr]- and [Glu]MTs in an epithelial cell line by microinjecting hapten-labeled brain tubulin and then assaying its pattern of incorporation. [Glu]MTs, as compared with [Tyr]MTs, turned over slowly and grew poorly. These results define two MT populations possessing different rates of turnover and suggest a relationship between the stability of cytoplasmic MTs and their tyrosination state.

MATERIALS AND METHODS

Cells and Tissue Culture. African green monkey kidney epithelial cells (TC-7) were cultured in Dulbecco's modified Eagle's medium containing 10% (vol/vol) fetal bovine serum as described (24).

Preparation of Hapten-Labeled Tubulin. Microtubule protein was isolated under conditions favoring tyrosination as described (33, 34). Tubulin was separated from microtubule-associated proteins by DEAE-Sephadex chromatography (35) and was then hapten-labeled with either dichlorotriazinylaminofluorescein (DTAF) (4) or *N*-hydroxysuccinimidyl biotin (36). The hapten-labeled material, after two cycles of temperature-dependent assembly and disassembly using 5% (vol/vol) dimethyl sulfoxide (37), was injected at a concentration of ≈4 mg/ml. [Glu]tubulin composition was low as determined by the failure to observe [Glu]MTs after injection into a test cell line (human fibroblasts) that lacks endogenous [Glu]MTs (5).

Microinjection and Immunofluorescence. Cells were microinjected as described (4) with DTAF or biotinylated tubulin, incubated for various times, lysed for 60 sec in extraction buffer containing 0.1% Triton X-100 (38), and fixed in extraction buffer with either 0.7% glutaraldehyde for 15 min or 5 mM ethylene glycol bis-(succinic acid *N*-hydroxysuccinimide ester) (Sigma) for 20 min.

Cells injected with DTAF-tubulin were stained sequentially with antibodies specific for the fluorescein hapten (39), [Glu]tubulin (24), and [Tyr]tubulin (40) (Accurate Chemicals Westbury, NY). Each MT subclass was labeled with fluorescein-conjugated secondary antibodies and was photographed individually before the next staining regimen was

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Abbreviations: DTAF, dichlorotriazinylaminofluorescein; [Glu]tubulin, α-tubulin with a carboxyl-terminal glutamic acid; MT, microtubule; [Tyr]tubulin, α-tubulin with a carboxyl-terminal tyrosine; [Glu]MT, detyrosinated MT; [Tyr]MT, tyrosinated MT.

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performed. Cells injected with biotinylated tubulin were incubated with Texas Red-conjugated streptavidin, the rabbit [Glu]tubulin antibody, the rat [Tyr]tubulin antibody, and fluorescein isothiocyanate-conjugated anti-rabbit IgG before photographing them. The coverslips were then restained with Texas Red-conjugated anti-rat IgG and photographed again. The streptavidin and secondary antibodies were purchased from Jackson ImmunoResearch (Avondale, PA). The proportions of each MT class were obtained from tracings of photographic prints as described (5). MT subclasses were distinguished either by the use of different fluorophores or by comparing photographs of each subclass and detecting nonoverlapping MTs.

RESULTS

Experimental Approach. Hapten-labeled brain tubulin was microinjected into mammalian tissue culture cells (TC-7). Analysis of the pattern of its incorporation into the MT network over time allowed us to examine the turnover of MT subsets within the same cell. MTs were first classified as either [Tyr]- or [Glu]MTs, based on their reactivity with subunit-specific antibodies. MTs may be considered in general to be uniform copolymers of [Tyr]- and [Glu]tubulin subunits and to vary in composition from mostly [Tyr]tubulin to mostly [Glu]tubulin (32). For the purposes of this study, we defined [Glu]MTs as those exceeding the minimum detection limit, which we estimate from an injection study (5) to correspond to a composition of ≈20% [Glu]tubulin. The [Glu]MT class would, therefore, include [Glu]MT/[Tyr]MT copolymers over a wide range of composition, except that MTs with <20% [Glu]tubulin would be classified as [Tyr]MTs. MTs that preexisted or had polymerized between the time of injection and the time of cell lysis were distinguished as those lacking or containing hapten-labeled subunits, respectively. This approach permitted us to monitor [Glu]MT turnover independently of the predominant [Tyr]MT array.

[Glu]MTs Do Not Grow Efficiently. TC-7 cells extracted and fixed within 2 min of microinjection displayed a dense pattern of short MT domains containing hapten-labeled subunits (Fig. 1*a*). Each [Tyr]MT with an observable end (≈300 ends in the cell in Fig. 1) displayed a domain of hapten-labeled subunits at its end (Fig. 1 *a*, *c*, *d*, and *f*). In contrast, only a few [Glu]MTs displayed segments of hapten-labeled tubulin at their ends (14% for biotin and 28% for DTAF-injected cells). Most of the MTs classified as [Glu]-MTs did not display hapten-labeled domains at their ends, and we, therefore, conclude that they did not grow.

[Tyr]MTs Turn Over Rapidly. After 1 hr of incubation, the pattern of hapten-labeled MTs in microinjected cells closely resembled the [Tyr]MT pattern (Fig. 2 *a* and *c*) but was different from the [Glu]MT array (Fig. 2*b*). At higher magnification, some copolymers of [Glu]tubulin and biotinylated tubulin were observed (Fig. 2 *d* and *e* arrowheads), whereas many [Glu]MTs displayed no biotin stainining. In contrast, all observed [Tyr]MTs contained biotinylated tubulin (Fig. 2 *d* and *f*), indicating that by 1 hr the entire [Tyr]MT population had turned over. Similar results were obtained with fluorescein-labeled tubulin.

[Glu]MTs Turn Over Slowly. The turnover of [Glu]MTs was examined quantitatively over time in an asynchronous population of interphase cells (Fig. 3). Within 2 min after injection, except for the end-labeling of a small proportion, [Glu]MTs lacked hapten-labeled tubulin. Cells examined 1, 8, and 16 hr after injection displayed a progressively smaller proportion of [Glu]MTs that had not incorporated hapten-conjugated subunits. Conversely, the proportion of [Glu]-MTs containing hapten-labeled tubulin increased over time, indicating that these MTs had become detyrosinated after microinjection and incorporation of hapten. The production of [Glu]MTs was biphasic, including an initial, relatively rapid rise followed by a much slower increase over a period of hours. Specifically, the proportion of hapten-labeled [Glu]MTs rose from 0 to 45% after 1 hr of incubation, and the slower rise corresponded to an increase from 45 to 80% over

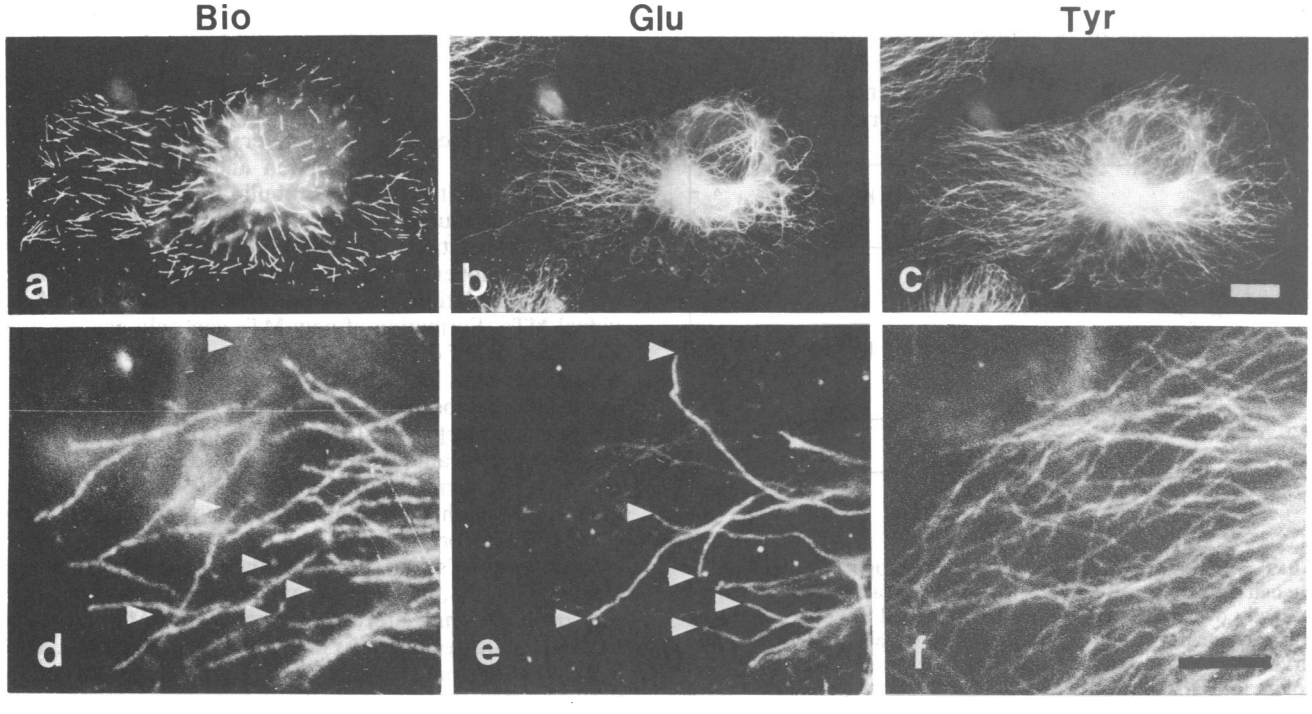

FIG. 1. Pattern of incorporation of biotinylated tubulin into [Tyr]- and [Glu]MTs within 2 min of microinjection. The distribution of biotinylated segments (Bio) (*a*) was similar to the distribution of [Tyr]MT ends (*c*) but was different from the distribution of [Glu]MT ends (*b*). Higher magnification images revealed contiguity between biotin segments (*d*) and [Tyr]MT ends (*f*) but not [Glu]MT ends (*e*). Arrowheads identify ends of [Glu]MTs (*e*) for which there are no corresponding biotin segments (*d*). [Bars: *a–c*, 10 μm; *d–f* (different cell), 5 μm.]

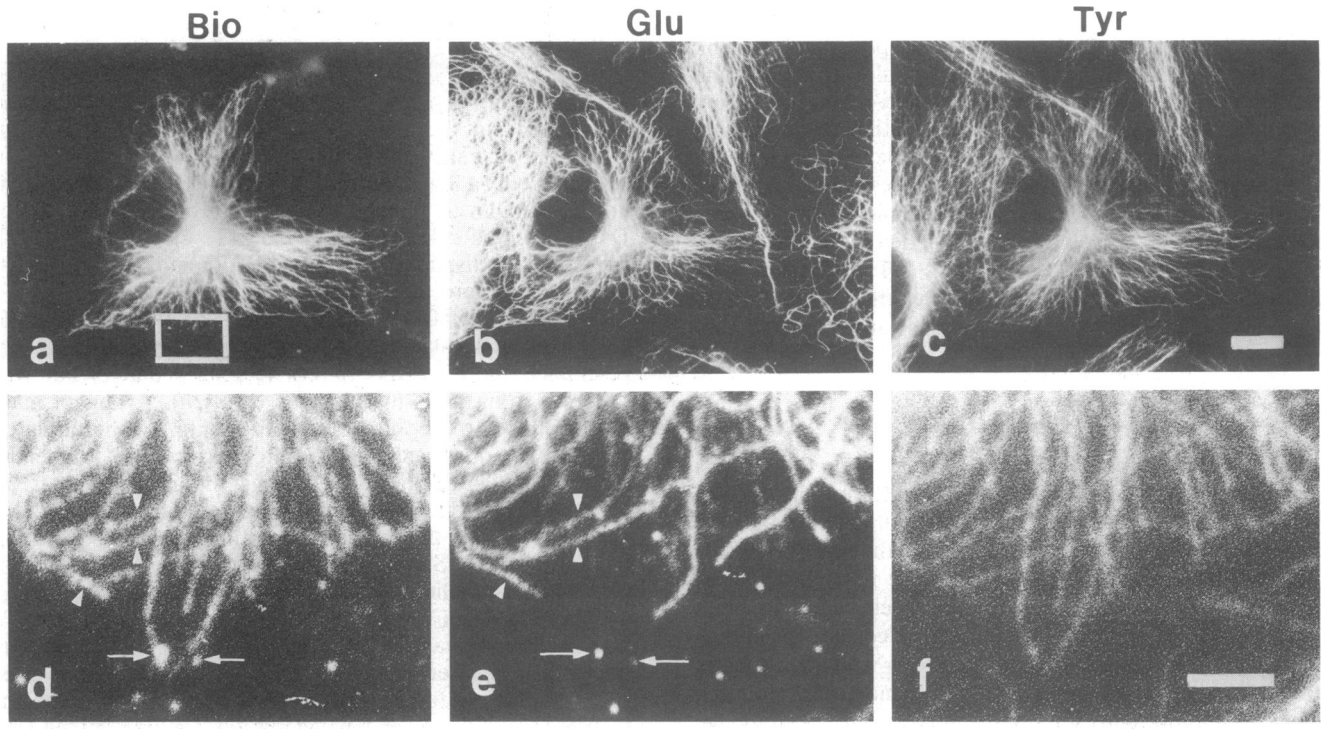

FIG. 2. Hapten-conjugated tubulin incorporates more rapidly into the [Tyr]MT population than the [Glu]MT population. Cells incubated for 1 hr after microinjection displayed a pattern of biotin staining (*a*) that closely resembled the [Tyr]MT network (*c*), though the [Glu]MT pattern was clearly different (*b*). A section of this cell (outlined in *a*) shown at higher magnification (*d–f*) demonstrates that biotin-containing MTs and [Tyr]MTs show a 1:1 numerical correspondence (*d* and *f*), but only a few [Glu]MTs have incorporated hapten-labeled subunits (*d* and *e*, arrowheads). The arrows (*d* and *e*) mark two biotinylated [Tyr]MTs that do not contain [Glu]tubulin. (Bars: *a–c*, 10 μm; *d–f*, 3 μm.)

the period from 1 to 16 hr after injection, an average increase of 2.3% per hr.

Mitosis Mixes All Tubulin Pools. MT patterns of cells that were injected during interphase and had entered or completed mitosis at the time of cell lysis (8–24 hr after injection) were also examined (Fig. 4). Though resolution of individual MTs in the mitotic spindle at the light microscopic level is not possible, the patterns of hapten-labeled tubulin and [Glu]- and [Tyr]MTs were indistinguishable (Fig. 4 *a–c*). After mitosis was completed and cells had reentered interphase, distributions of individual [Glu]- and [Tyr]MTs in daughter cells could again be determined (Fig. 4 *e*, *f*, *h*, and *i*). Although [Glu]- and [Tyr]MT patterns were not identical, uniform staining for hapten-conjugated subunits was found in all MTs of both populations (compare Fig. 4*d* with *e* and *f*, and *g* with *h* and *i*), indicating that all stable, interphase [Glu]MTs had disassembled and that all daughter-cell MTs were formed from a common pool containing a homogeneously distributed proportion of hapten-labeled subunits.

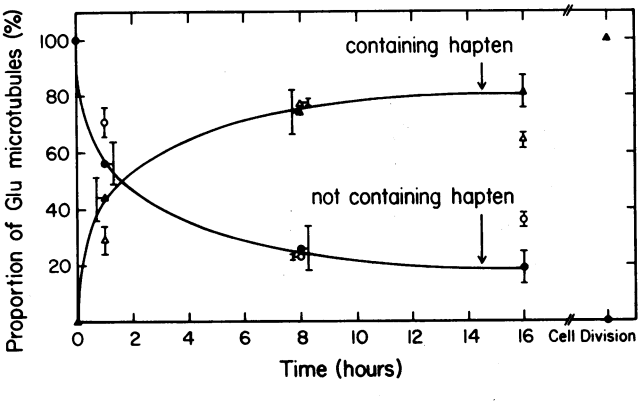

FIG. 3. [Glu]MTs turn over slowly. The proportion of [Glu]MTs that had not exchanged subunits with the cytoplasmic pool decreased gradually over time. [Glu]MTs were classified as either containing (triangles) or not containing (circles) hapten-labeled subunits. The curve was drawn using the biotin experiments only (solid symbols), though the DTAF data is also presented (open symbols). Values along the abscissa represent incubation times after microinjection and do not correspond to cell-cycle stages. There was no significant difference between the biotin and DTAF values for any time point except at 16 hr, using Student's *t* test of independent samples ($P < 0.05$). Cells in mitosis did not contain MTs without hapten.

DISCUSSION

Previous analyses of MT turnover measured the dynamic behavior of the bulk population and were made without regard to [Glu]tubulin/[Tyr]tubulin composition. In this study we measured the turnover rate of the [Glu]- and [Tyr]MT populations separately. In the dynamic instability model of turnover, the steady state is defined as the balance, over the population, between growth and shrinking of individual MTs. Formation of new MTs can only occur if there is breakdown of old MTs. Therefore, incorporation of hapten-labeled MTs was taken as an indicator of MT turnover and lack of labeling was taken as an indicator of MT persistence. The [Tyr]MT population turned over significantly within 5 min and completely within 1 hr of injection. In contrast, the turnover of the [Glu]MT population required many hours, with some remaining unlabeled for most of a cell cycle (≈22 hr for TC-7 cells).

Most [Glu]MTs did not support the end addition of hapten-labeled subunits, whether assayed immediately after injection or 10–20 min later, when many [Tyr]MTs contained hapten-conjugated subunits along their entire lengths. These [Glu]MTs were not growing, even at a rate much slower than their [Tyr]MT counterparts. Therefore, [Glu]MTs were not only resistant to breakdown (one component of turnover) but also did not grow appreciably (the other component). Since we have demonstrated (5) the ability of [Glu]tubulin subunits to add to the end of [Tyr]MTs, the failure of [Glu]MTs to

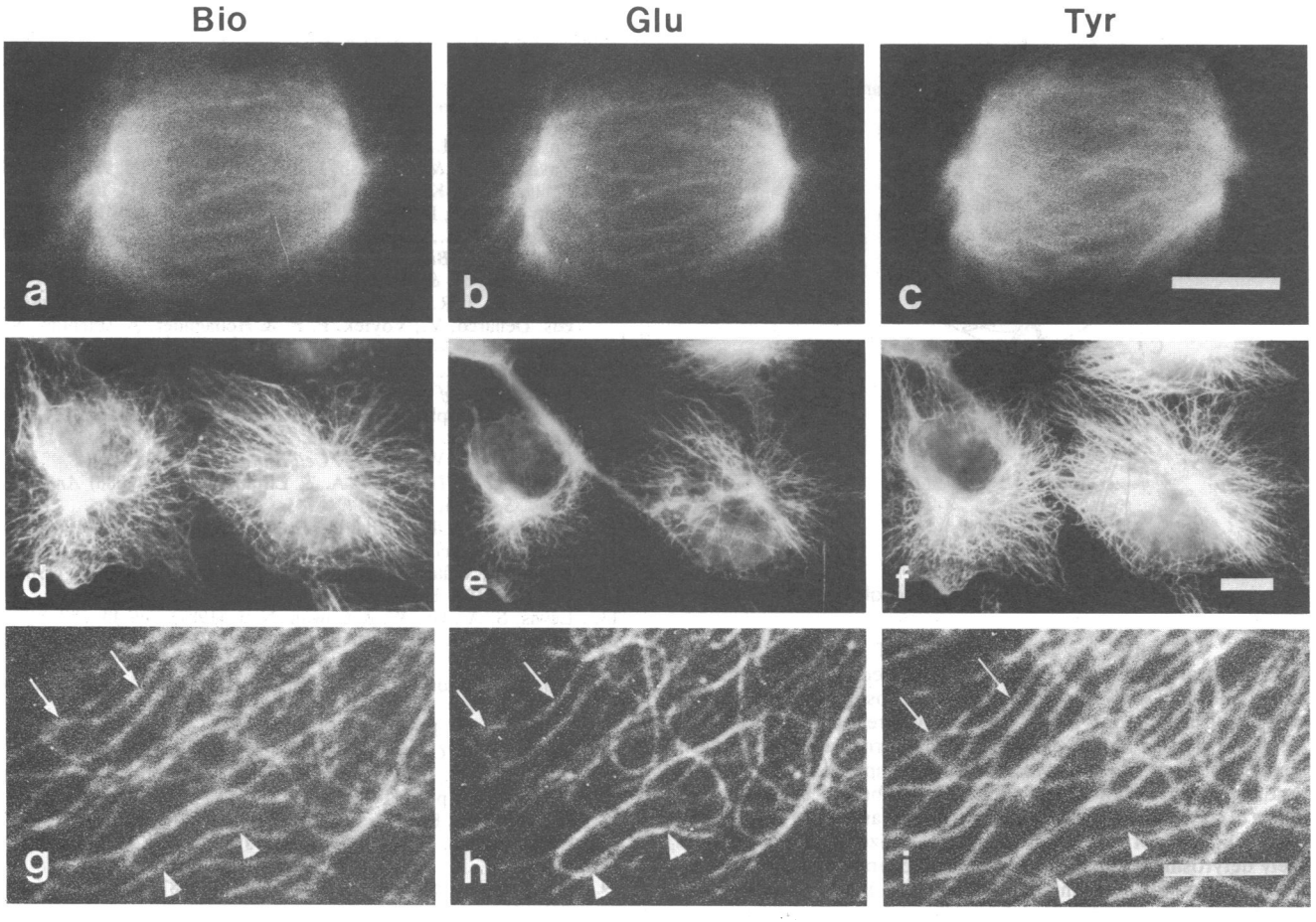

FIG. 4. Mitosis mixes all tubulin pools. Microinjected cells were not inhibited from entering mitosis (*a–c*), and the staining patterns of biotinylated subunits (*a*), [Glu]tubulin subunits (*b*), and [Tyr]tubulin subunits (*c*) were indistinguishable. Daughter cells (*d–f*) contained biotinylated subunits (*d*) in [Glu]MTs (*e*) and [Tyr]MTs (*f*). This is shown at higher magnification in another cell (*g–i*). The biotin staining (*g*) was evenly distributed between both highly detyrosinated MTs (*g–i*, arrowheads) and highly tyrosinated MTs (*g–i*, arrows). (Bars: *a–c* and *g–i*, 5 μm; *d–f*, 10 μm.)

elongate suggests that they may be "capped" or structurally less efficient as templates for addition of subunits.

Although we identified a subpopulation of MTs by a minimum level of detyrosination, it is likely that MTs within that group contained various concentrations of [Glu]tubulin. It is possible that the [Glu]MTs that supported end addition of subunits and the [Glu]tubulin–hapten-conjugated tubulin copolymers that were observed after 1 hr of incubation actually contained [Glu]tubulin levels close to the minimum detection limit and possessed turnover properties more closely associated with [Tyr]MTs than [Glu]MTs.

The presence of some MTs containing [Glu]tubulin and hapten-labeled subunits indicated that they assembled after microinjection. Since the soluble tubulin pool in TC-7 cells contains virtually no [Glu]tubulin dimers (41), these MTs must have assembled as copolymers of [Tyr]- and hapten-labeled tubulin that gradually became detyrosinated over time. This interpretation is in agreement with the finding that MT detyrosination is a post-polymerization modification (41).

We may ask to what extent the production of new [Glu]MTs and the persistence of old ones can be accounted for in terms of known rates of [Tyr]MT turnover and detyrosination. For the purposes of calculation, we will assume half-times for [Tyr]MT turnover and detyrosination of 10 min and ≈40 min (23), respectively. The former value predicts that 1 hr (six half-times) after microinjection of hapten, ≈1.5% of the MTs (6–8 MTs in an average TC-7 cell containing 400–500 MTs) would not yet have turned over if MT depolymerization was due only to a first-order decay process. These remaining MTs would have become sufficiently detyrosinated during the 1-hr period to be scored as hapten-free [Glu]MTs. By this model, after 16 hr the probability of a cell containing a hapten-free [Glu]MT would be vanishingly small ($P < 10^{-28}$). However, we observed 25–50 hapten-free [Glu]MTs per cell throughout the 16-hr postinjection period—orders of magnitude in excess of the predictions of this simple kinetic model. Therefore, these MTs, identified by their content of [Glu]tubulin, represent a stable subclass of the bulk MT population.

The production of [Glu]MTs (Fig. 3) was biphasic. The initial increase in [Glu]MTs containing hapten is predicted by the simple kinetic model and probably represents the incorporation of hapten through the rapid turnover of [Tyr]MTs followed by their detyrosination. Thus, most of these [Glu]-MTs containing hapten would be expected to be [Glu]tubulin–[Tyr]tubulin copolymer of varying composition, depending upon time after polymerization. Were these copolymers "[Tyr]tubulin-like" in their dynamics, they would turn over rapidly leading to a steady-state low level of [Glu]MTs, all containing hapten. However, the progressive, slow increase in hapten-labeled [Glu]MTs, complementary to the slow loss of hapten-free [Glu]MTs, is not predicted by the simple model and suggests that an additional process may contribute to [Glu]MT formation. This additional process may result in MTs that are mostly [Glu]tubulin that very slowly, if at all, break down during interphase.

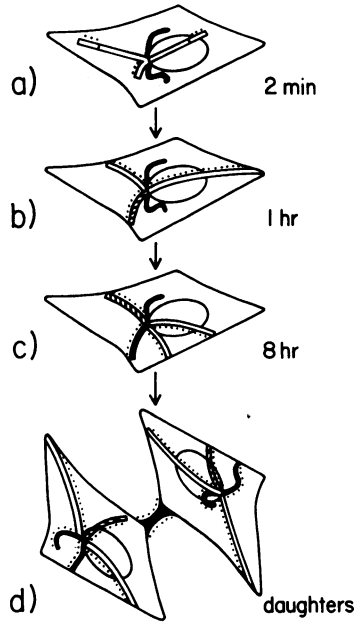

FIG. 5. A model for the mechanism of [Glu]MT turnover. Important features are that only [Tyr]MTs (open MTs) add subunits at their ends and grow directly from the centrosome and, therefore, incorporate hapten (dots on MT surface), whereas [Glu]MTs cannot (*a*). All [Tyr]MTs are accessible to the detyrosinating activity of tubulin carboxypeptidase, which is slow compared with [Tyr]MT turnover. Most [Tyr]MTs break down rapidly but, by chance, a few escape disassembly long enough to be detected as [Glu]MTs (hatched MT in *b*). Some of these MTs become stabilized and more extensively detyrosinated (dotted, solid MTs in *c*) and replace [Glu]MTs that occasionally depolymerize. When cells divide all interphase MTs depolymerize, and, when a new interphase array is formed, all MTs contain hapten-labeled subunits (*d*).

However, cells injected in interphase that later entered cell division showed uniform hapten labeling. The nonidentical [Glu]- and [Tyr]MT patterns of daughter cells, presumably generated by detyrosination after reentry into interphase, each contained hapten-labeled subunits, indicating that the stable [Glu]MTs present during the previous cell cycle had depolymerized. Therefore, the maximum duration of any stable [Glu]MT was one cell cycle.

In summary, the cytoplasmic [Glu]MT population turns over more slowly than the [Tyr]MT population (Fig. 5). New [Glu]MTs are added to the population by detyrosination of aging [Tyr]MTs. Some [Glu]MTs are depleted from the population throughout the cell cycle by the normal turnover mechanism, while some remain stable throughout interphase and disassemble only as the cell enters mitosis. Second-generation cells, having completed mitosis and reshuffled all tubulin pools, continue the cycle of tyrosination/detyrosination and selective stabilization.

Although the mechanism that generates functionally distinct MT populations within a cell remains a puzzle, we have identified a correlation between diminished dynamic activity and an elevated level of detyrosination. It remains to be determined whether detyrosination confers stability to MTs or arises as a consequence of an independent stabilizing mechanism.

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