Postpolymerization Detyrosination of α-Tubulin: A Mechanism for Subcellular Differentiation of Microtubules

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Abstract. Tyrosinated (Tyr) and detyrosinated (Glu) α-tubulin, species interconverted by posttranslational modification, are largely segregated in separate populations of microtubules in interphase cultured cells. We sought to understand how distinct Tyr and Glu microtubules are generated in vivo, by examining time-dependent alterations in Tyr and Glu tubulin levels (by immunoblots probed with antibodies specific for each species) and distributions (by immunofluorescence) after microtubule regrowth and stabilization. When microtubules were allowed to regrow after complete depolymerization by microtubule antagonists, Glu microtubules reappeared with a delay of ∿25 min after the complete array of Tyr microtubules had regrown. In these experiments, Tyr tubulin immunofluorescence first appeared as an aster of distinct microtubules, while Glu tubulin staining first appeared as a grainy pattern that was not altered by detergent extraction, suggesting that Glu microtubules were created by detyrosination of Tyr microtubules. Treat-

ments with taxol, azide, or vinblastine, to stabilize polymeric tubulin, all resulted in time-dependent increases in polymeric Glu tubulin levels, further supporting the hypothesis of postpolymerization detyrosination. Analysis of monomer and polymer fractions during microtubule regrowth and in microtubule stabilization experiments were also consistent with postpolymerization detyrosination; in each case, Glu polymer levels increased in the absence of detectable Glu monomer. The low level of Glu monomer in untreated or nocodazole-treated cells (we estimate that Glu tubulin comprises <2% of the monomer pool) also suggested that Glu tubulin entering the monomer pool is efficiently retyrosinated. Taken together these results demonstrate that microtubules are polymerized from Tyr tubulin and are then rapidly converted to Glu microtubules. When Glu microtubules depolymerize, the resulting Glu monomer is retyrosinated. This cycle generates structurally, and perhaps functionally, distinct microtubules.

T YROSINATION of tubulin is an unprecedented posttranslational modification. In an ATP-requiring reaction, a tyrosine residue is added to the COOH terminus of the α-subunit of tubulin (Barra et al., 1973, 1974). This unusual reaction is carried out by a single, highly specific enzyme, tubulin tyrosine ligase (Raybin and Flavin, 1977a; Murofushi, 1980). However, as encoded by most α-tubulin genes, newly synthesized α-tubulin contains a COOH-terminal tyrosine residue (e.g., Valenzuela et al., 1981; Villasante et al., 1986). Thus, the primary modification is usually the removal of the COOH-terminal tyrosine, and this is carried out by a second enzyme, tubulin carboxypeptidase (Argarana et al., 1978), which is also highly specific for α-tubulin (Kumar and Flavin, 1981). Therefore, depending on the COOH-terminal residue of the α-subunit, tubulin exists in two forms: tyrosinated or detyrosinated. (We have abbreviated these Tyr[1] or Glu, referring to their respective COOH-terminal residues.)

The function of this unique posttranslational modification of tubulin remains unknown, although many studies have implicated it in alterations of the cytoskeleton, especially during differentiation. In *Xenopus* embryos, there is an increase in the amount of tubulin that can be tyrosinated (i.e., Glu tubulin) during early development (Preston et al., 1981). Similarly, an increase in the levels of Glu tubulin accompany brain development in the chick (Rodriguez and Borisy, 1978) and the rat (Rodriguez and Borisy, 1979; Barra et al., 1980). In the study of Rodriguez and Borisy (1979), the increase in Glu tubulin paralleled a decrease in the specific activity of the ligase. Alterations in the ligase activity have also been described during chick brain and muscle development (Deanin et al., 1977). Consistent with these biochemical studies is a more recent study showing that immunoreactivity with a Tyr tubulin–specific mAb is progressively lost from parallel fiber axons in the cerebellar cortex during brain maturation (Cumming et al., 1984).

The rates of posttranslational tubulin tyrosination have been measured during shape changes in a number of cell types. Increased tyrosination rate has been observed in Chi-

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nese hamster ovary (CHO) cells treated with dibutyryl cAMP (Deanin et al., 1981) and after stimulation of rabbit and human leukocytes with chemoattractants (Nath et al., 1981; 1982). The absolute rates of tyrosination measured in these studies suggest a relatively rapid turnover of COOH-terminal tyrosine. In cultured muscle cells, the half-life of COOH-terminal tyrosine was 37 min, whereas α-tubulin half-life was >48 h (Thompson et al., 1979). Thus, alterations in tyrosination (or detyrosination) may effect rapid changes in the behavior of tubulin and microtubules.

Tyr and Glu tubulin do not differ dramatically in their behavior in vitro. The rate and extent of polymerization appear to be identical for each species (Raybin and Flavin, 1977b; Arce et al., 1978; Kumar and Flavin, 1982). Similarly, only subtle differences in the binding of microtubule-associated proteins have been described (Kumar and Flavin, 1982).

Because of the importance of cellular localization for the function of tubulin, and the lack of any obvious difference in the in vitro behavior of the two species, we suspected that the function of tyrosination/detyrosination might involve, in part, a differential localization of the two species. Accordingly, we examined the distribution of the Tyr and Glu forms of tubulin using antibodies specific for each species and found that they were distributed in a heterogeneous pattern in the microtubules of cultured cells: some microtubules contained predominantly Tyr tubulin, while adjacent microtubules were composed primarily of Glu tubulin (Gundersen et al., 1984). This surprising result cannot be explained by a simple incompatibility of the two species to form copolymers, since individual microtubules formed in vitro from mixtures of Glu and Tyr monomers appear to reflect the composition of the monomer pool (our unpublished observations). Given the rapid turnover of microtubules in cultured cells (Saxton et al., 1984, Schulze and Kirschner, 1986), one would predict a rapid homogenization of the two forms, rather than the segregation we observed.

Because of the likelihood that structurally different microtubules have functionally distinct roles, we wanted to determine the mechanism by which distinct Glu and Tyr microtubules are created. In this report we use our specific antibodies to follow the progress of the posttranslational modifications as they occur in their cellular context. We find that an array of distinct Tyr and Glu microtubules can be rapidly generated after the removal of agents that have completely disrupted the original array. Combined with an analysis of the distributions of the two species in the monomer and polymer pools and the effect of microtubule-stabilizing agents on the number of Tyr and Glu microtubules, our results establish a cyclic mechanism by which the two populations of microtubules can be created. Such a cycle may provide a paradigm for understanding the rapid cytoskeletal changes involved in cellular morphogenesis.

Materials and Methods

Cell Culture and Treatments

TC-7 cells, cultured in DME supplemented with 10% FBS (growth medium) as previously described (Gundersen et al., 1984), were seeded onto glass coverslips (for immunofluorescence) or plastic petri dishes (for preparation of SDS extracts) and allowed to grow for 2–3 d before commencing an experiment. Unless noted otherwise, all treatments involving cells were carried out at 37°C. All drugs were prepared as stock solutions in DMSO and

added to growth medium such that the final DMSO concentration did not exceed 0.1% (vol/vol). This concentration of DMSO had no effect on the distribution or levels of Tyr or Glu tubulin in cells (data not shown).

For microtubule regrowth experiments, endogenous microtubules were first depolymerized by treating cells with one of the following: 4 μM nocodazole (Aldrich Chemical Co., Milwaukee, WI) for 4 h; 4 μM Colcemid (demecolcine; Sigma Chemical Co., St. Louis, MO) for 4 h; or 0°C for 1 h. For the cold treatment, growth medium at 0°C was added to the dish of cells; the dish was subsequently placed on an ice-water bath in the refrigerator. To permit the regrowth of microtubules, cells in nocodazole or Colcemid were rinsed twice with medium without added drugs and then placed into fresh growth medium. The time of addition of the second wash was taken as the zero time point. Cold treatment was reversed by removing the medium from the cells and adding fresh growth medium at 37°C. The time of addition of the warm medium was taken as zero time in this case. After release, cells were placed in a 37°C incubator and, at various intervals, samples were prepared for immunofluorescence as described below.

To stabilize microtubules, cells were treated with 5 μM taxol (a gift from Dr. Matthew Suffness, Natural Products Branch, Division of Cancer Treatment, National Cancer Institute), 10 μg/ml vinblastine sulfate (Aldrich Chemical Co.) or 20 mM sodium azide (Sigma Chemical Co.). The taxol and vinblastine treatments were performed in growth medium; the azide treatment was conducted in PBS containing 0.8 mM $MgCl_2$ and 0.7 mM $CaCl_2$ (Dulbecco's PBS) (Bershadsky and Gelfand, 1981). At appropriate times, cells were prepared for immunofluorescence or were dissolved in SDS sample buffer for immunoblot analysis (see below).

Preparation of Monomer and Polymer Fractions

Monomer and polymer fractions were prepared from taxol-treated cells and from cells recovering from nocodazole treatment (both as described above). We initially followed the procedure of Caron et al. (1985), extracting the cells with 0.1% Triton X-100 in a microtubule stabilization buffer for 30 min. However, with this long extraction, we detected substantial conversion of Tyr to Glu tubulin in the polymer fraction (data not shown). Accordingly, we modified the extraction conditions to limit this spontaneous conversion of Tyr to Glu tubulin. Cells on 100-mm plastic petri dishes were rapidly rinsed twice in microtubule-stabilizing buffer (MSB) (85 mM Pipes, pH 6.93, 1 mM EGTA, 1 mM $MgCl_2$, 2 M glycerol, and protease inhibitors [10 μg/ml aprotinin, 0.5 mM benzamidine, 5 μg/ml o-phenanthroline, and 0.2 mM phenylmethylsulfonyl fluoride]), and then extracted with 1.6 ml of MSB containing 0.5% (vol/vol) Triton X-100. After 3 min, the Triton extract (monomer fraction) was gently removed to a graduated tube, the volume was noted and 1/5 volume of 5× SDS buffer (10% SDS, 325 mM Tris-HCl, pH 6.8, 30% glycerol, 250 mM DTT and 1 mM phenylmethylsulfonyl fluoride) was added. This sample was then boiled for 5 min. The cytoskeletons remaining on the plate were carefully rinsed once with MSB and then solubilized with 1.6 ml MSB plus 0.4 ml 5× SDS buffer. After 5 min, this extract (polymer fraction) was removed from the plate and boiled 5 min. Samples were stored at −70°C until analyzed by immunoblotting (see below).

For most experiments, we visually checked the extent of extraction. Cells on coverslips were extracted as above, but instead of solubilizing the cytoskeletons, the extracted cells were fixed in −20°C methanol for 5 min and then processed for immunofluorescence.

Indirect Immunofluorescence

Cells grown on glass coverslips were fixed after the treatments described above by briefly rinsing them in Earle's balanced salt solution and then plunging them into −20°C methanol. As noted above, extracted cytoskeletons were rinsed in MSB before methanol fixation. The fixed cells or cytoskeletons were rehydrated in 10 mM Tris-buffered saline, pH 7.4 (TBS), and if not used immediately, stored in TBS plus 0.1% sodium azide at 6°C.

The fixed cells (or cytoskeletons) were stained by double indirect immunofluorescence using a rabbit peptide antibody specific for Glu tubulin (Gundersen et al., 1984) and a rat mAb (designated YL1/2) specific for Tyr tubulin (Kilmartin et al., 1982; Wehland et al., 1983). YL1/2 was the generous gift of Dr. J. V. Kilmartin (Medical Research Council, Cambridge). Dilutions were prepared in TBS with 10% normal goat serum. The Glu antibody was used at a 1/50 dilution of antiserum; YL1/2 was used at a 1/250–1/500 dilution of an ascites fluid. Staining of the Tyr tubulin array identical to that observed with YL1/2 was obtained with a rabbit peptide antibody specific for Tyr tubulin (Gundersen et al., 1984) (data not shown).

Second antibodies were fluorescein-conjugated goat anti-rat IgG and rhodamine-conjugated goat anti-rabbit IgG (obtained from Cooper Biomedical Inc., Malvern, PA) and were used at 1/25 dilutions. No difference

was observed if the fluorochromes were switched. Incubations with the Glu and Tyr antibodies were done simultaneously, as were subsequent incubations with the second antibodies. Fluorescence microscopy and photography were performed as previously described (Gundersen et al., 1984).

Immunoblots

For the preparation of whole cell samples, (from taxol-, azide-, and vinblastine-treated cells), cells were solubilized from 100-mm dishes in 2 ml SDS sample buffer (2% SDS, 100 mM Tris-HCl, pH 6.8, 25% glycerol, 0.1% bromphenol blue, 50 mM DTT, and 0.2 mM phenylmethylsulfonyl fluoride) and boiled 5 min. Samples were subjected to electrophoresis on 7.5% SDS polyacrylamide gels (Laemmli, 1970) and then transferred to nitrocellulose sheets (2 h at 9 V/cm in the transfer buffer of Towbin et al., 1979). After the blocking step, blots were reacted with either the Tyr or Glu rabbit antibodies (both diluted 1/5,000), followed by a 1/1,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG (Cooper Biomedical Inc.). 4-Chloro-1-naphthol was used as the chromagen.

Quantification of Tyr and Glu Tubulin

To quantify the levels of Tyr and Glu tubulin, duplicate samples subjected to electrophoresis on SDS polyacrylamide gels were immunoblotted along with varying amounts of porcine brain tubulin as a standard. The brain tubulin was prepared directly from brain extract by one-step DEAE chromatography, without cycles of polymerization-depolymerization, essentially as described by Murphy (1982), and was >95% pure as judged by quantitative densitometry of SDS polyacrylamide gels. After development, immunoblots were dried in the dark and then scanned with a video densitometer (model No. 620, Bio-Rad Laboratories, Richmond, CA) in the reflectance mode. Because the intensity varied across individual bands, each band was scanned at six different positions and the values were averaged. For both Tyr and Glu tubulin, this assay was linear between 40 and 200 ng of tubulin and had a limit of sensitivity of ~20 ng.

Brain tubulin is a mixture of Tyr and Glu tubulin (e.g., see Rodriguez and Borisy, 1979); therefore, to use brain tubulin as an absolute standard, it was necessary to determine the levels of the two forms in brain tubulin. This was accomplished by determining the level of Glu immunoreactivity of brain tubulin before and after treatment with carboxypeptidase A (CPA). CPA treatment removes the preexisting COOH-terminal tyrosine residues without digesting the tubulin further (e.g., see Raybin and Flavin, 1977b) and thus yields pure Glu tubulin that can be used to determine the proportion of Glu tubulin in brain tubulin. The Tyr tubulin is then assumed to represent the remainder of the tubulin. For convenience, the CPA digestion was performed after samples of brain tubulin had been transferred to nitrocellulose from SDS polyacrylamide gels, although digestion of samples before electrophoresis gave identical results. After the usual blocking step, nitrocellulose sheets were rinsed five times in 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, and then incubated with rocking for 30 min in 50 ml of 10 μg/ml CPA, pretreated with phenylmethylsulfonyl fluoride (Worthington Biochemical Corp./Cooper Biomedical Inc., Malvern, PA), in the same buffer at 37°C. Complete conversion to Glu tubulin was confirmed by the absence of immunoreactivity with the Tyr antibody. As determined with this assay, the level of Glu tubulin in DEAE brain tubulin was 52.4 ± 1.5% (n = 3).

Protein determination was with a modified Lowry assay (Geiger and Bessman, 1972) using BSA as a standard. Samples in SDS buffer were prepared for protein assay as described by Tornqvist and Belfrage (1976).

Results

Throughout this study we have used TC-7 cells, an epithelial cell line derived from African green monkey kidney, whose distributions of Tyr and Glu tubulin have been described (Gundersen et al., 1984). As in many cultured cells, most of the microtubules in interphase TC-7 cells are stained only with an antibody specific for Tyr tubulin (Tyr antibody) (Fig. 1 a), while a few are stained only with an antibody to Glu tubulin (Glu antibody) (Fig. 1 b). In addition some microtubules are stained brightly with both antibodies. We will refer to microtubules stained brightly with the Tyr antibody as Tyr microtubules and those stained brightly with the Glu antibody as Glu microtubules. This classification is primarily a
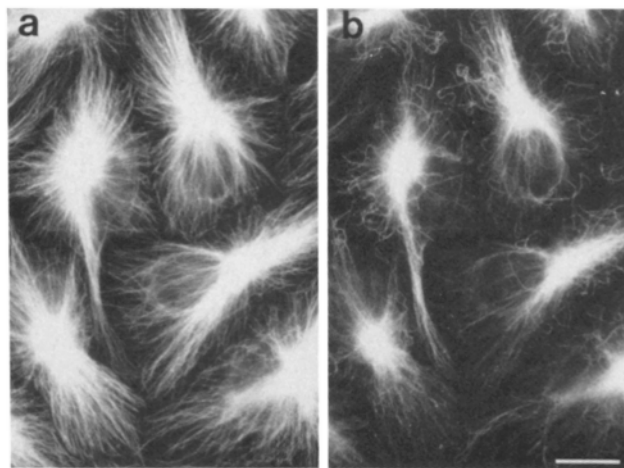

Figure 1. Distribution of Tyr and Glu tubulin in TC-7 cells. Methanol-fixed cells were double stained with the rat Tyr and rabbit Glu antibodies followed by fluorescein-conjugated goat anti-rat IgG and rhodamine-conjugated goat anti-rabbit IgG. Fluorescence images of typical cells stained with (a) Tyr antibody and (b) Glu antibody. Bar, 20 μm.

matter of convenience and by using it we do not mean to imply that there are microtubules that are composed of only Tyr or only Glu tubulin. In fact, immunolocalization at the ultrastructural level suggests that nearly all microtubules contain some level of each species (Geuens et al., 1986). Most of the results we report here have been confirmed with at least one other cell line in addition to the TC-7 cells; they are, thus, probably indicative of the behavior of microtubules in most cultured cells.

Reappearance of Tyr and Glu Tubulin in Microtubules Regrown In Vivo

To determine if the heterogeneous distribution of Tyr and Glu tubulin in cellular microtubules could be regenerated from a pool of monomeric tubulin, we conducted microtubule regrowth experiments in vivo, similar to those performed earlier by others (e.g., Osborn and Weber, 1976; DeBrabander et al., 1981a). We also reasoned that the patterns of recovery of the two forms might yield information as to how the separate populations of microtubules were created. To initiate the experiment, endogeneous microtubules were completely depolymerized by treating cells with either cold or drugs, such as nocodazole or Colcemid; the resultant pool of tubulin monomer was then allowed to polymerize by rewarming the cells or removing the drugs. Fig. 2 shows an example of a typical regrowth experiment, in which cells were first treated with nocodazole and then the nocodazole was washed away. The nocodazole treatment depolymerized almost all cellular microtubules (Fig. 2, a and b); a few short microtubule remnants were still observed in some cells (Fig. 2 b). In addition, a single dot near the nucleus was brightly stained with the Glu antibody and only dimly stained with the Tyr antibody (see also Fig. 3); we have previously shown that this dot of staining corresponds to the centrosome (Gundersen and Bulinski, 1986a, b). The Tyr staining of nocodazole-treated cells was bright, but diffuse (Fig. 2 a), suggesting that the monomeric tubulin result-
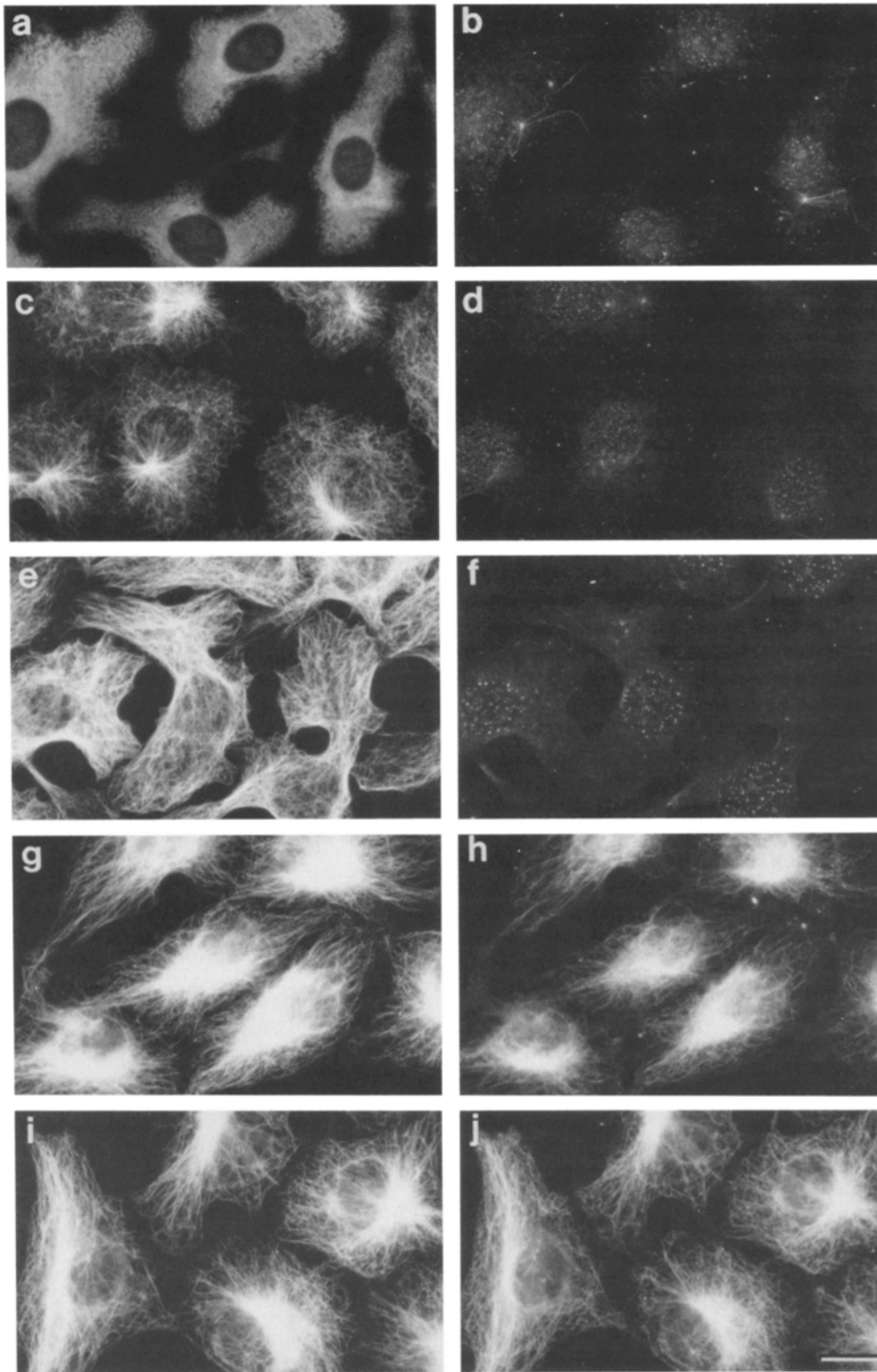
Figure 2. Microtubule regrowth in vivo after the release of cells from nocodazole treatment. Cells were treated with nocodazole to depolymerize endogenous microtubules and then the nocodazole was washed away to permit microtubule regrowth. Cells were fixed at the indicated times and then double stained with the Tyr (*a, c, e, g,* and *i*) and Glu (*b, d, f, h,* and *j*) antibodies and appropriate second antibodies. (*a* and *b*) Cells after 4 h of 4 μM nocodazole treatment (0-time point); (*c* and *d*) 3 min after nocodazole removal; (*e* and *f*) 7 min after nocodazole removal; (*g* and *h*) 30 min after nocodazole removal; (*i* and *j*) 80 min after nocodazole removal. Bar, 20 μm.
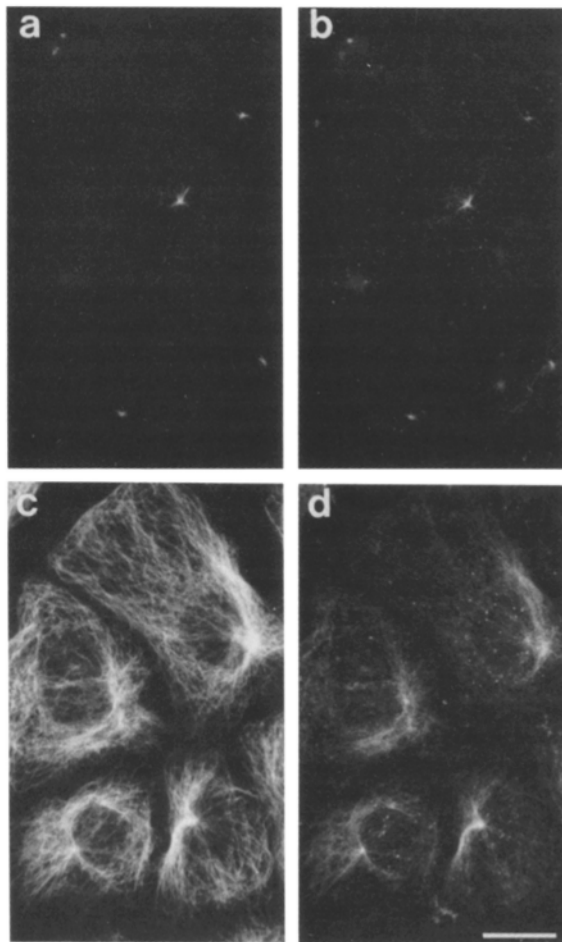
ing from the depolymerization of microtubules was primarily in the Tyr form.

When cells were released from nocodazole, microtubules rapidly reformed. Initially, both nucleated (centrosomal) and nonnucleated (free) microtubules formed (Fig. 2 *c*), but as growth continued the number of free microtubules decreased and centrosomal microtubules predominated (Fig. 2, *e, g,*

and *i*). This result is similar to the regrowth of microtubules in vivo described earlier (Osborn and Weber, 1976; DeBrabander et al., 1981*a*); however, from the standpoint of the present study, the most striking result is that the newly formed microtubules were stained only with the Tyr antibody. This was true for both the centrosomal and free microtubules found 3 min after release from nocodazole (Fig.

2, c and d) and for the completely regenerated array of microtubules observed 7 min after release (Fig. 2, e and f). While the Tyr pattern did not change significantly during the remainder of the time course, other than to become more focused to the centrosome (Fig. 2, g and i), the Glu staining of microtubules increased. Distinct Glu microtubules (i.e., those stained throughout their length with the Glu antibody) were first observed ~30 min after removal of nocodazole (Fig. 2 h). At this time, the array of Glu microtubules was similar to that observed in untreated cells (compare Fig. 2 h with Fig. 1 b). However, the increase in Glu tubulin continued for 1–2 h, surpassing the level in untreated cells (compare Fig. 2 j with Fig. 1 b; see also Fig. 10 below).

In contrast to the observed pattern of recovery of Tyr microtubules, we did not observe an aster of Glu microtubules at any time during the recovery. Between the time when cells were devoid of Glu staining (up to 7 min) and the time when distinct Glu microtubules reappeared (at 30 min), we did observe a progressive increase in Glu staining; however, the pattern was grainy and diffuse, rather than confined to an

Table I. Recovery Times for Tyr and Glu Microtubule Arrays

Depolymerization treatment*	Reappearance of Tyr array‡	Reappearance of Glu array§	Δ(Glu-Tyr)
	min	*min*	
Nocodazole (4 μM, 4 h)	7	30	23
Cold (0°C, 1 h)	3	25	22
Colcemid (4 μM, 4 h)	50	80	30

* Cells were treated to depolymerize all endogenous microtubules, and then microtubules were allowed to regrow by removing the drug (nocodazole or Colcemid) or by adding warm growth medium.
‡ The time at which a Tyr-stained microtubule array, filling the cells, was first detected by immunofluorescence.
§ The time at which distinct Glu-stained microtubules were first detected by immunofluorescence.

Figure 3. Distribution of Tyr and Glu tubulin in detergent-extracted cells before and after microtubule regrowth. Cells were treated as in Fig. 2, but before fixation and staining, cells were extracted with Triton X-100 to remove monomeric tubulin (see text). Cells were then double stained with the Tyr (a and c) and Glu (b and d) antibodies and appropriate second antibodies. (a and b) Nocodazole-treated cells (0-time point); (c and d) 10 min after nocodazole removal. Bar, 20 μm.

aster of distinct, short microtubules. To determine whether this hazy staining was due to an increase in polymeric or monomeric Glu tubulin, we extracted cells recovering from nocodazole, under conditions that stabilize polymeric tubulin (see Materials and Methods and below). The extraction protocol used efficiently removed monomeric tubulin (compare Fig. 3 a with Fig. 2 a) without noticeably perturbing the distribution of polymeric tubulin (compare Fig. 3 c with Fig. 2 e). In the example shown in Fig. 3, we have compared cells treated with nocodazole (0-min time point) (Fig. 3, a and b) with cells released from nocodazole for 10 min (Fig. 3, c and d). A significant increase in the amount of Glu staining between the 0 and 10 min time points can be seen. The grainy Glu staining is observed throughout the cell, appearing brighter in those areas of high microtubule density (compare Fig. 3, c and d). Since these cells were extracted before fixation and staining, this suggests that the grainy Glu staining observed at intermediate times is due to Glu tubulin residing in microtubules. Further experiments addressing this point are described below.

We have also performed in vivo regrowth experiments in which cells were released from treatments with cold or Colcemid, to determine if the timing and pattern of reappearance of Tyr and Glu tubulin were unique to cells released from nocodazole. We found the same patterns of regrowth shown in Fig. 2 for release from either cold or Colcemid; however, the elapsed times required for recovery of the microtubule arrays were different (see Table I). Nonetheless, the time between the reappearance of a fully regrown array of Tyr microtubules (e.g., as shown in Figure 2 e) and the initial reappearance of distinct Glu microtubules (e.g., as shown in Fig. 2 h) was very similar for all three types of regrowth experiments (Table I). This was the case even though the cytoplasmic array of microtubules was regenerated over different time courses, requiring up to 50 min to form after Colcemid release (Table I). Thus, Glu microtubules reappeared with a characteristic delay of ~25 min after the polymerization of Tyr microtubules.

The in vivo microtubule regrowth experiments show that the normal distribution of Tyr and Glu microtubules can be rapidly generated from a pool of monomeric tubulin. The preponderance of Tyr staining in nocodazole-treated cells (Fig. 2 a) and also in the microtubules newly regrown after release from nocodazole (Fig. 2, c and e) both suggest that
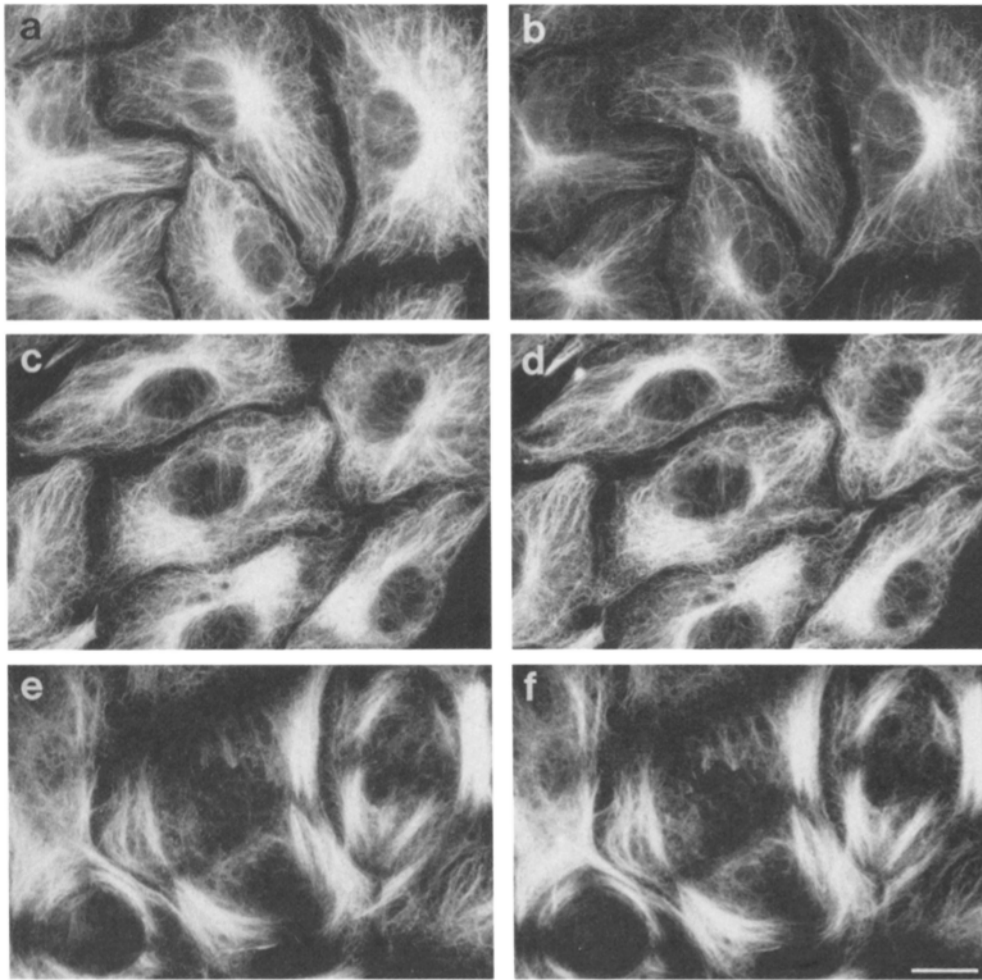
Figure 4. Effect of taxol on the distribution of Tyr and Glu tubulin. Cells were treated with 5 µM taxol, fixed at the indicated times, and then double stained with Tyr (a, c, and e) and Glu (b, d, and f) antibodies as described in the legend to Fig. 1. Fluorescence images from cells treated with taxol for (a and b) 15 min; (c and d) 60 min; and (e and f) 24 h. Bar, 20 µm.

the pool of monomer is composed primarily of Tyr tubulin. This implies that Glu microtubules are created from pre-existing Tyr microtubules, rather than being formed de novo from monomeric Glu tubulin. The characteristic delay in the reappearance of Glu microtubules and the hazy pattern of Glu staining observed at intermediate times of regrowth support this notion. A postpolymerization conversion of Tyr to Glu tubulin would also be consistent with the preference of tubulin carboxypeptidase for polymeric tubulin in in vitro assays (Kumar and Flavin, 1981; Arce and Barra, 1985). We have tested this putative mechanism in more detail in the experiments described below.

Effect of Microtubule-stabilizing Agents on Tyr and Glu Tubulin Distribution

If Glu microtubules are formed by the detyrosination of Tyr microtubules, then agents that act to stabilize tubulin in the polymeric form should result in an increase in the level of Glu tubulin in microtubules. We tested this idea by treating cells with taxol, a drug that dramatically lowers the critical concentration for microtubule polymerization and acts to stabilize microtubules in vivo (Schiff et al., 1979; Schiff and Horwitz, 1980; DeBrabander et al., 1981a, b). We observed a significant increase in the level of Glu staining in microtu-

bules as early as 15 min after the addition of taxol to TC-7 cells (compare Fig. 4 b to Fig. 1 b); little difference in the array of microtubules stained with the Tyr antibody could be detected at this time (Fig. 4 a). By 60 min of treatment the patterns of Tyr and Glu staining were virtually indistinguishable (Fig. 4, c and d). In addition to its effects on the critical concentration of tubulin, taxol induces bundling of microtubules in vivo (Schiff and Horowitz, 1980; DeBrabander et al., 1981a, b). In the TC-7 cells used in this study, bundling was first noted at 60 min (Fig. 4, c and d); thus, the increase in Glu tubulin preceded the bundling effect of taxol. Similarly, microtubules not yet incorporated into bundles after 60 min of taxol treatment still showed elevated levels of Glu staining. Longer taxol treatments (2–24 h; Fig. 4, e and f) resulted in increased bundling of microtubules as observed by others (DeBrabander et al., 1981a, b), making it difficult to discern further increases in Glu tubulin levels in individual microtubules. Given the rapid increase in the level of Glu tubulin in response to taxol, we were surprised to find that there was still significant Tyr staining of taxol-induced microtubule bundles at 24 h (Fig. 4 e).

To confirm that taxol treatment resulted in an actual increase in Glu tubulin levels, we performed immunoblot analysis of whole cell samples prepared at different times of taxol treatment. Throughout this study we probed immunoblots
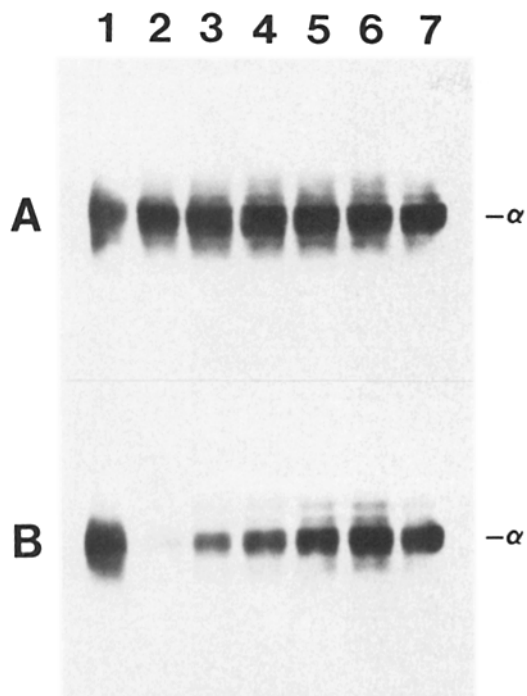
Figure 5. Immunoblot analysis of taxol-treated cells. SDS samples were prepared from cells treated with 5 μM taxol for various times and then subjected to electrophoresis on SDS–polyacrylamide gels. A constant volume of extracted sample was loaded on each lane; this yielded equivalent protein loads as judged from Coomassie-stained gels (data not shown). After transfer to nitrocellulose, blots were probed with the rabbit Tyr (*A*) or Glu (*B*) antibodies. (Lanes *1*) Brain tubulin standard; (lanes *2*) untreated cells; (lanes *3–7*) cells treated with taxol for 15 min, 30 min, 60 min, 4 h, and 24 h, respectively. The position of the α-tubulin band determined from Coomassie-stained gels is shown on the right (*α*).

with rabbit Tyr and Glu antisera at dilutions that yielded approximately equal levels of immunoreactivity towards samples of DEAE-purified brain tubulin (Fig. 5, *A* and *B*, lane *1*). Since this brain tubulin is composed of 48% Tyr and 52% Glu tubulin (see Materials and Methods), such a control ensures that we are detecting the two forms at approximately equal sensitivities. Results of an immunoblot analysis of taxol-treated cells were consistent with the immunofluorescence data; Glu tubulin was detectably elevated within 15 min of taxol addition, and longer treatments resulted in higher Glu tubulin levels until ~4 h, when the level of Glu tubulin reached a plateau (Fig. 5 *B*). Little difference in the Tyr tubulin levels was observed during the taxol treatment (Fig. 5 *A*). The final level of Glu tubulin was approximately equal to that of Tyr tubulin and was maintained up to 24 h (Fig. 5, *A* and *B*, lanes *6* and *7*). These results demonstrate that immunofluorescence staining accurately reflects increases in Glu tubulin levels caused by taxol.

The increase in Glu tubulin levels in the taxol-treated cells was significant for two reasons. (*a*) It showed that the level of Glu tubulin in cells was not fixed, but could be altered rapidly. (*b*) It further supported the hypothesis that Glu microtubules are generated after polymerization. Presumably, by stabilizing microtubules, taxol gives the tubulin carboxypeptidase a longer time to work on the subunits of an individual microtubule. To extend these results, we investigated the effects of azide (in glucose-free medium) and vinblastine on the level and distribution of Tyr and Glu tubulin. Although these agents have dramatically different modes of action, they both effect a stabilization of tubulin in a polymeric form. Azide, by depleting cellular ATP, blocks an unidentified step in the in vivo depolymerization of microtubules (Moskalewski et al., 1980; Bershadsky and Gelfand, 1981; DeBrabander et al., 1981*a*), and thus acts to stabilize cellular
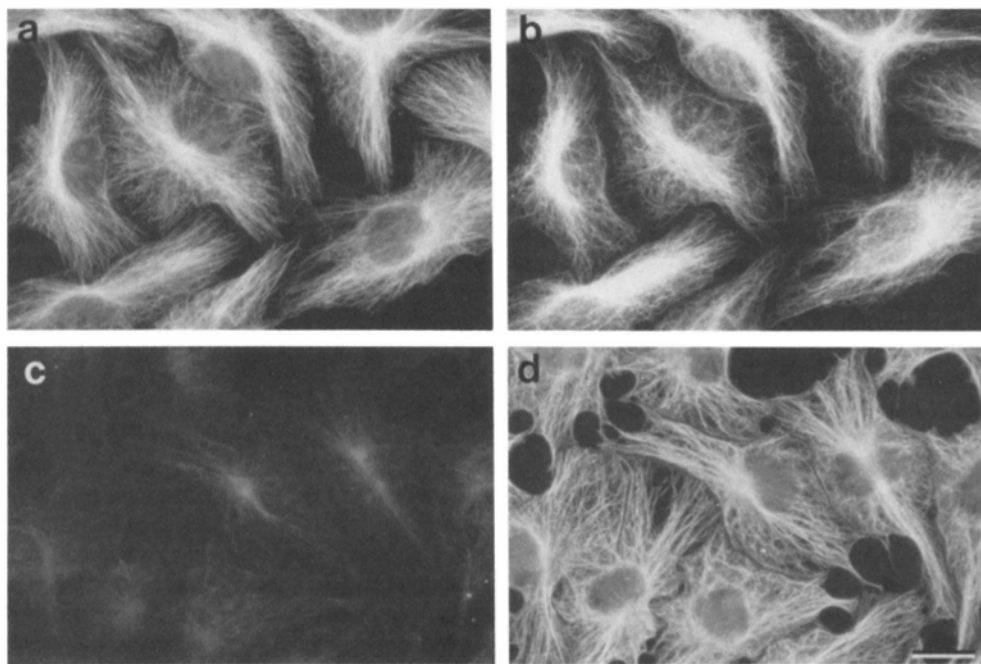
Figure 6. Effect of azide on the distribution of Tyr and Glu tubulin. Cells were treated with 20 mM sodium azide (in glucose-free medium), fixed at various times, and then double stained with the Tyr (*a* and *c*) and Glu (*b* and *d*) antibodies as in the legend to Fig. 1. Immunofluorescence images of cells treated with azide for (*a* and *b*) 30 min and (*c* and *d*) 3 h. Bar, 20 μm.
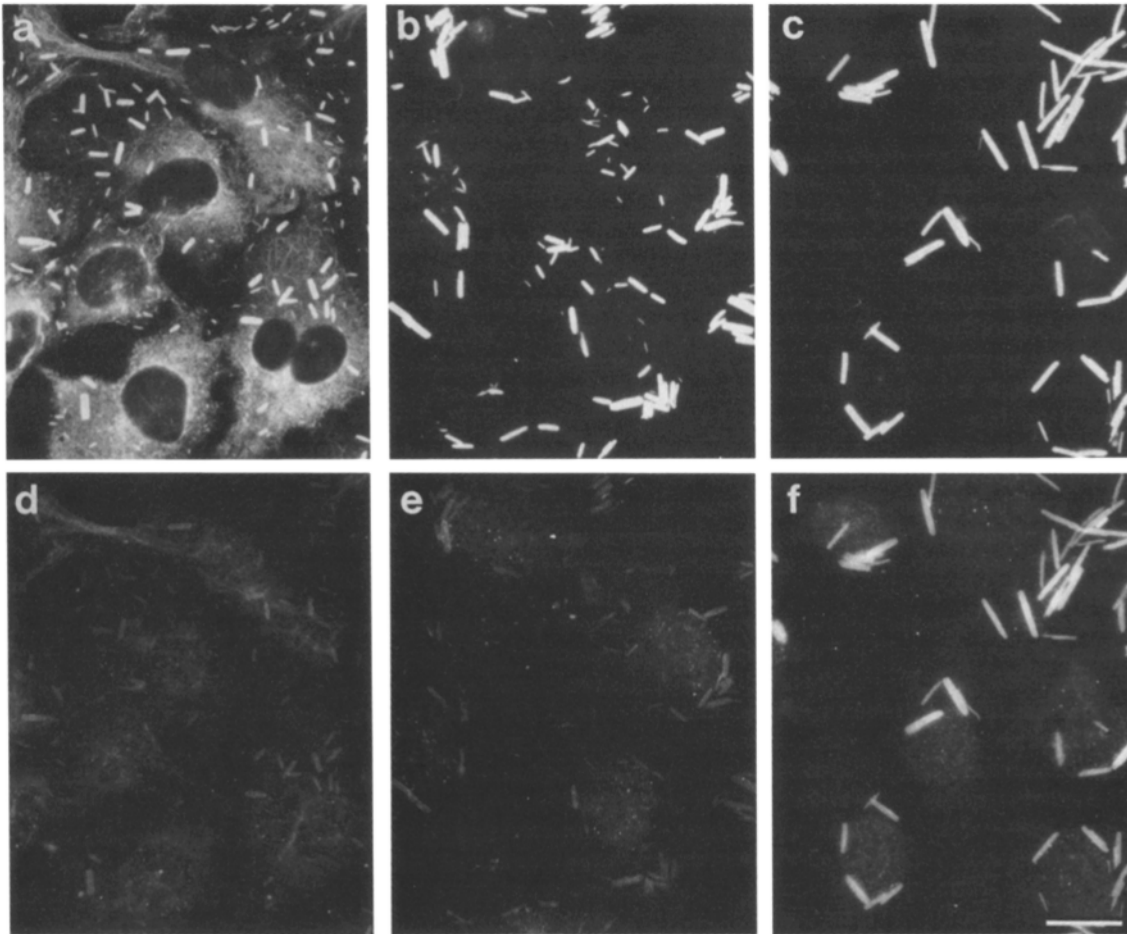
Figure 7. Effect of vinblastine on the distribution of Tyr and Glu tubulin. Cells were treated with 10 µg/ml vinblastine sulfate, fixed at various times, and then double stained with Tyr (*a–c*) or Glu (*d–f*) antibodies (see Fig. 1). Immunofluorescence images of cells treated with vinblastine for (*a* and *d*) 15 min; (*b* and *e*) 60 min; and (*c* and *f*) 12 h. Bar, 20 µm.

microtubules. On the other hand, vinblastine initially depolymerizes all the endogeneous microtubules and subsequently drives the incorporation of the monomeric tubulin into paracrystalline bundles of protofilamentous tubulin (Dustin, 1984); thus, vinblastine also stabilizes tubulin in a polymeric form.

As shown in Figs. 6 and 7, azide and vinblastine both caused an increase in the level of Glu staining. By 30 min of azide treatment the number of microtubules labeled with the Glu antibody was clearly elevated (Fig. 6 *b*; for comparison, see untreated cells in Fig. 1 *b*). Little effect on the Tyr staining of microtubules was observed at this early time of treatment (Fig. 6 *a*). Longer azide incubations resulted in further increases in Glu staining, so that by 2 h almost every microtubule was labeled with both the Glu and Tyr antibodies (data not shown) and by 3 h only the Glu antibody labeled microtubules brightly (Fig. 6, *c* and *d*). As described by others (Moskalewski et al., 1980; Bershadsky and Gelfand, 1981; DeBrabander et al., 1981*a*), we found that azide treatment stabilized microtubules against depolymerization by drugs (data not shown).

Vinblastine also caused an increase in Glu staining; however, the results are complicated slightly by the fact that the initial effect of vinblastine is to rapidly depolymerize all of the endogenous microtubules; by 15 min of vinblastine treat-

ment very few microtubules remained (Fig. 7, *a* and *d*). The bright, but diffuse, staining of cells with the Tyr antibody at this time (Fig. 7 *a*), analogous to the Tyr staining of nocodazole-treated cells (Fig. 2 *a*), presumably reflects the presence of monomeric (Tyr) tubulin. Formation of the paracrystalline bundles of tubulin occurred shortly after microtubule breakdown; at 15 min, the nascent crystals were stained brightly only with the Tyr antibody (Fig. 7, *a* and *d*). By 60 min, diffuse Tyr staining disappeared, and the paracrystals increased in size (Fig. 7 *b*), but they still did not exhibit significant Glu staining (Fig. 7 *e*). With longer vinblastine treatments (>4 h), all of the paracrystals showed increased staining with the Glu antibody (Fig. 7, *c* and *f*).

As with the taxol treatment, we confirmed that the increase in Glu staining of azide- and vinblastine-treated cells was not the result of unmasking of Glu epitopes or some other artifact associated with the immunofluorescence protocol. Immunoblots of the azide time course (Fig. 8, *A* and *B*) showed an increase in the cellular level of Glu tubulin by 30 min of azide treatment (Fig. 8 *B*, lane *4*). Longer treatments resulted in further increases in Glu tubulin levels, which paralleled decreases in the levels of Tyr tubulin so that by 3 h, Glu tubulin was the predominant form (Fig. 8). An analogous increase in Glu tubulin levels was detected on immunoblots of vinblastine-treated cells; however, unlike taxol or azide treat-
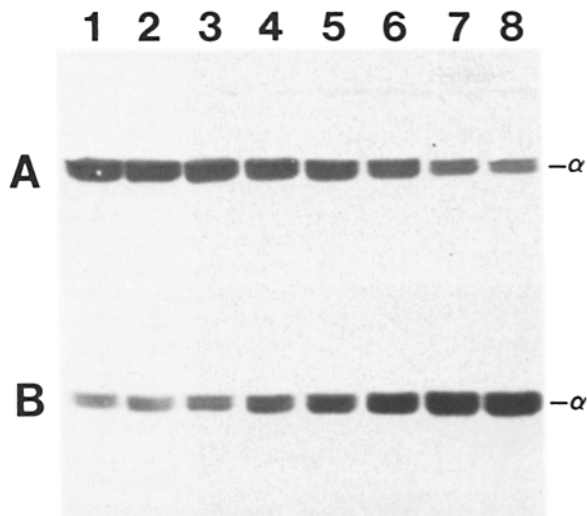
Figure 8. Immunoblot analysis of azide-treated cells. Samples prepared from cells treated with 20 mM azide in glucose-free medium were analyzed by immunoblotting (as in legend to Fig. 5). Blots containing equivalent amounts of protein were probed with the rabbit Tyr (A) or Glu (B) antibodies. (Lane 1) Untreated cells; (lanes 2–8) cells treated with azide for 5 min, 15 min, 30 min, 60 min, 2 h, 3 h, and 4 h, respectively. The position of the α-tubulin band determined from Coomassie-stained gels is shown on the right (α).

Glu and Tyr tubulin to create distinct Glu and Tyr microtubules, was not ruled out. To examine critically the contributions of these two possible pathways for the creation of Glu-rich microtubules, we have prepared monomer and polymer fractions of tubulin by detergent extraction of cells. By this approach, we were also able to assess the dynamics of Tyr and Glu tubulin in the monomer pool, information that could not be obtained from immunofluorescent staining.

To separate the monomer and polymer pools of Tyr and Glu tubulin, we followed a protocol similar to that described by Caron et al. (1985), in which cells are extracted with Triton X-100 in a microtubule-stabilization buffer. However, we used a higher Triton concentration and shorter extraction times (see Materials and Methods) in order to limit the spontaneous enzymatic conversion of Tyr to Glu tubulin that we observed during extraction (see below). As assessed by immunofluorescence, the extraction conditions we used did not appear to alter appreciably the level of polymer or leave significant amounts of unextracted monomer in the cells (see Fig. 3). The lack of contaminating monomer in the polymer pool is even more convincingly demonstrated by immunoblots of cells treated with nocodazole to break down all of the microtubules; while no tubulin was detected in the polymer fraction (see Fig. 10, A and B, lane 11), quantification showed that 95% of the total cell tubulin was recovered in the monomer pool. Whether polymeric tubulin was released into the monomer pool is more problematic to ascertain; such a release could occur during the extraction by detachment of cells, by fragmentation of microtubules, or by dilution-induced depolymerization of labile microtubules. As mentioned above, immunofluorescence of the extracted cells showed no dramatic loss of microtubules during extraction. Also, the lack of detectable tubulin in the monomer fraction prepared from taxol-treated cells (Fig. 9 A and B), which would be expected to contain predominantly polymeric tubulin (Schiff et al., 1979, 1980), suggests that few cells detached during the extraction and that stabilized microtubules were not fragmented or depolymerized. However, at this time, we cannot rule out the possibility that depolymerization of some labile microtubules occurred in experiments in which the cells were not previously exposed to taxol.
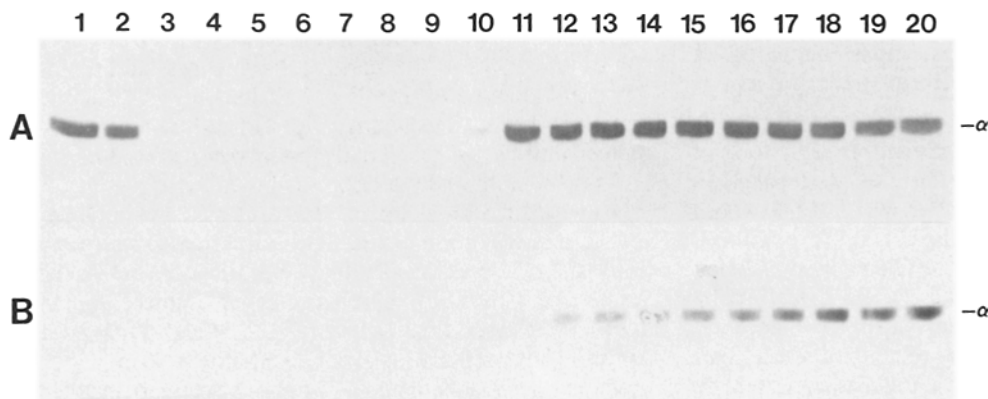
ments, the increase was significant only after long (>4 h) treatments (data not shown). Thus, for each treatment, the amount of Glu and Tyr tubulin detected on immunoblots corroborated the levels of Glu and Tyr staining observed by immunofluorescence.

Levels of Tyr and Glu Tubulin in Monomer and Polymer Pools

Both the regrowth and stabilization experiments strongly suggest that Glu tubulin is generated from Tyr tubulin while in the polymeric state. However, an alternative hypothesis, that Glu monomer is first created from Tyr monomer and is then polymerized into (Glu) microtubules, although seemingly unlikely because it would require a complex sorting of

Figure 9. Immunoblot analysis of Tyr and Glu tubulin levels in monomer and polymer fractions prepared from taxol-treated cells. Monomer and polymer fractions from taxol-treated cells were subjected to electrophoresis on SDS–polyacrylamide gels, applying extracts from the same number of cells to each lane of the gel (as verified by Coomassie staining, each lane of the monomer blot and each lane of the polymer blot contained equal amounts of protein [data not shown]). After transfer to nitrocellulose, the blots were probed with the rabbit Tyr (A) or Glu (B) antibodies. (Lanes 1 and 11) Whole-cell extract from untreated cells; (lanes 2) monomer fraction from untreated cells; (lanes 3–10) monomer fraction from cells treated with taxol for 2 min, 5 min, 10 min, 15 min, 30 min, 2 h, 6 h, and 12 h, respectively; (lanes 12) polymer fraction from untreated cells; (lanes 13–20) polymer fraction from cells treated with taxol for 2 min, 5 min, 10 min, 15 min, 30 min, 2 h, 6 h, and 12 h, respectively. The position of the α-tubulin band determined from Coomassie-stained gels is shown on the right (α).
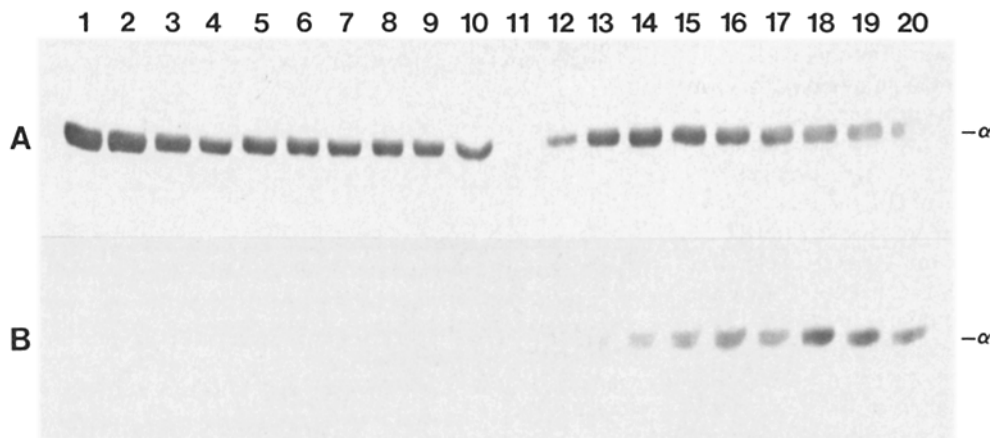
Figure 10. Immunoblot analysis of Tyr and Glu tubulin levels in monomer and polymer fractions during microtubule regrowth in vivo. Cells were treated with nocodazole to depolymerize endogenous microtubules and then the nocodazole was washed out to permit microtubule regrowth. Monomer and polymer fractions, prepared at the indicated times, were subjected to electrophoresis on SDS–polyacrylamide gels, applying extracts from an equivalent number of cells to each lane of the gel (as verified by Coomassie staining, each lane of the monomer blot and each lane of the polymer blot contained equal amounts of protein [data not shown]). To maximize the detection of Glu tubulin, three times as much monomer or polymer extract was loaded on the gel to be stained with the Glu antibody compared with that to be stained with the Tyr antibody. After transfer to nitrocellulose, blots were probed with the rabbit Tyr (*A*) or Glu (*B*) antibodies and developed with peroxidase. Monomer fractions from cells released from nocodazole treatment for (lanes *1*) 0 min; (lanes *2*) 2.5 min; (lanes *3*) 5 min; (lanes *4*) 7.5 min; (lanes *5*) 10 min; (lanes *6*) 15 min; (lanes *7*) 30 min; (lanes *8*) 60 min; (lanes *9*) 2 h; and (lanes *10*) 3 h. (Lanes *11–20*) Polymer fractions from cells released from nocodazole treatment for 0 min, 2.5 min, 5 min, 7.5 min, 10 min, 15 min, 30 min, 60 min, 2 h, and 3 h, respectively. The position of α-tubulin determined from Coomassie-stained gels is shown on the right (α).

In our initial experiments, we sought to determine the levels of Tyr and Glu tubulin in monomer and polymer fractions prepared from untreated cells. To quantify the levels of the two forms, we scanned immunoblots (similar to those in Fig. 9, *A* and *B*) by reflectance densitometry, using purified brain tubulin to generate a standard curve for each experiment (see Materials and Methods). Using this method, we found that tubulin (the sum of the individual determinations of Tyr and Glu tubulin) comprised 3.5 ± 0.5% (*n* = 5) of the total TC-7 cell protein; other assays have yielded similar values in cultured cells (e.g., Hiller and Weber, 1978).

The quantitative analysis of Tyr and Glu tubulin levels in whole cells and in monomer and polymer fractions is summarized in Table II. Glu tubulin comprised 4.9% of the tubulin in whole cells and 4.5 ± 4.3% and 16.2 ± 3.1% of the tubulin recovered in the monomer and polymer fractions, respectively. The large standard deviation in the value for Glu monomer was the result of variability in its detection; in two of five experiments, we did not detect Glu monomer. These values for Glu monomer and polymer are clearly artifactually elevated above those that must exist in the cell, as shown by the greater-than-twofold increase in the amount of Glu tubulin recovered in the combined monomer and polymer fractions (Table II). This increase in the total Glu tubulin level cannot be due to preferential loss of Tyr tubulin, because 94% of the total cell tubulin was recovered in the two fractions (Table II). We attribute this extraction-induced increase in Glu tubulin to the activity of endogenous tubulin-specific carboxypeptidase; this enzyme, by generating Glu tubulin from Tyr tubulin, would increase Glu tubulin levels without altering the total amount of tubulin recovered. The level of monomeric Glu tubulin is probably further exaggerated due to the breakdown of labile microtubules; in nocodazole-treated cells, which contained virtually no microtubule polymer, we did not detect Glu monomer, and we estimate an even lower level of Glu monomer (see Discussion). Thus, although we cannot assign absolute values for Glu tubulin levels in the two fractions, our determinations represent upper limits and suggest that Glu tubulin is substantially enriched in microtubules.

As in untreated cells, the level of Glu monomer was very low in cells treated with taxol (Fig. 9 *B*, lanes *3–10*) and in cells recovering from nocodazole (Fig. 10 *B*, lanes *1–10*); in fact, we did not detect Glu monomer in two trials of each

Table II. Tyr and Glu Tubulin Levels in Whole Cells and Monomer and Polymer Fractions

| | Protein | Tubulin | Tubulin Composition | |
			Tyr	Glu
	%	%	%	%
Whole cell	(100)	(100)	95.1 ± 0.9	4.9 ± 0.9
Monomer	61 ± 2.2	36 ± 4.1	95.5 ± 4.3	4.5 ± 4.3
Polymer	39 ± 2.2	64 ± 4.1	83.8 ± 3.1	16.2 ± 3.1
Total (monomer + polymer)	99 ± 7.0	94 ± 5.5	87.3 ± 6.8	233 ± 35

The values represent the mean ± standard deviation from five separate experiments.
Monomer and polymer values were calculated from the actual amounts recovered (e.g., % monomer = monomer/monomer + polymer × 100).
Total (monomer + polymer) values were the totals recovered in comparison to the whole cell level (e.g., % total (monomer + polymer) = monomer + polymer/whole cell × 100).

type of experiment. Significantly, cells treated with nocoda-zole for 4 h showed no Glu monomer even though virtually all of the cellular tubulin detectable was in monomeric form (Fig. 10, lanes *1*). This suggests that upon depolymerization by nocodazole, the Glu tubulin that was originally in poly-mer was retyrosinated by tubulin tyrosine ligase. It also sup-ports the idea that our determination of Glu monomer in un-treated cells is artifactually elevated (see Discussion).

The low level of Glu tubulin in the monomer pool suggests that polymerization of Glu tubulin does not contribute significantly to the increases in Glu tubulin seen after taxol treatment or nocodazole release. The alternative hypothesis, the postpolymerization modification of Tyr to Glu tubulin, is further supported by the time course of Glu tubulin ac-cumulation in polymer. In taxol-treated cells, the level of monomeric tubulin decreased very rapidly, falling below de-tectable levels between 2 and 5 min after taxol addition (Fig. 9 *A*, lanes *2* to *10*); a corresponding increase in polymeric tubulin, while not obvious in Fig. 9 *A*, lanes *12–20*, was documented by blots in which the protein loads were lower than in this comparison. Significantly, the increase in poly-meric Tyr tubulin occurred within 2 min of taxol addition (Fig. 9 *A*, lane *13*); while a slight increase in Glu polymer was detected at 2 min, the majority of the increase occurred after much longer treatments (Fig. 9 *B*, lanes *12–20*).

The lag between the appearance of Tyr and Glu tubulin in microtubules was even more evident in cells released from nocodazole. As expected, the nocodazole treatment resulted in the complete depolymerization of microtubules; neither tubulin species was detected in the polymer fraction (Fig. 10, *A* and *B*, lane *11*) and quantitative densitometry showed that 95% of the cell tubulin was recovered in the monomer frac-tion. Upon nocodazole release there was a rapid polymeriza-tion of Tyr tubulin, so that by 5–10 min a plateau level of Tyr polymer was reached (Fig. 10 *A*, lanes *12–20*). The mono-mer pool, which apparently consisted only of Tyr tubulin, decreased in a fashion complementary to the increase in the polymer fraction (Fig. 10 *A*, lanes *1–10*). Polymeric Glu tubulin was not detected until 5 min after release, and in-creased over the next hour (Fig. 9 *B*, lanes *12–18*). Thus the major increase in Glu polymer levels occurred after the Tyr tubulin had already been incorporated into microtubules. In-terestingly, the level of Glu tubulin at time points ≥1 h after nocodazole release was greater than that seen in untreated cells (compare Fig. 10 *B*, lanes *18–20* with Fig. 9 *B*, lane *11*). This was also observed by immunofluorescence (see above). We have not determined precisely when cells recover from this "overshoot" in their content of Glu tubulin although the level appeared to drop after ~3 h (Fig. 10 *B*, lanes *19* and *20*).

Discussion

A Cyclic Model for the Posttranslational Tyrosination-Detyrosination of Tubulin

Our results suggest that tubulin is alternately detyrosinated and retyrosinated in vivo, and that the modifications occur to distinct pools of tubulin. Taken together with previous ex-periments which demonstrated the in vitro substrate specific-ities of the ligase (Arce et al., 1978) and the carboxypeptidase (Kumar and Flavin, 1981; Arce and Barra, 1985), our data
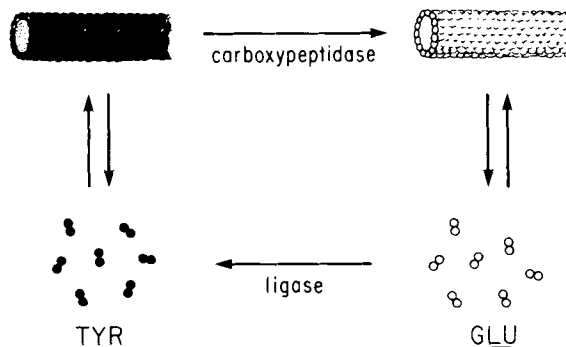
Figure 11. Cyclic model for the tyrosination–detyrosination of α-tubulin.

establish the cycle depicted in Fig. 11. We propose that Tyr monomer polymerizes to give Tyr microtubules; these are then acted upon by the tubulin carboxypeptidase to create Glu microtubules. When a Glu microtubule depolymerizes, the resulting Glu monomers are retyrosinated by the tubulin ligase to complete the cycle. As shown in Fig. 11, the poly-merization–depolymerization steps are envisioned to be re-versible; this reflects the ability of both species to polymerize and depolymerize in vitro (our unpublished observations; Raybin and Flavin, 1977b; Kumar and Flavin, 1982). The cy-clic nature of the model results from the restriction of the two enzymatic activities to different pools of tubulin, as dis-cussed in detail below. The possible cyclic nature of tyrosina-tion–detyrosination was first recognized in a treadmilling model proposed by Thompson (1982).

That Tyr tubulin polymerizes to yield Tyr microtubules seems fairly obvious: Tyr microtubules are the predominant type in the cell, and only Tyr tubulin was detected in the microtubules that had formed at the earliest stages we exam-ined in regrowth experiments (e.g., after nocodazole release; see Figs. 2 and 10). It might be argued that recovery from drugs (or cold) is unusual in that the cells are in a state of convalescence; however, we have also observed that only Tyr microtubules are formed in the "natural" regrowth of inter-phase microtubules after mitosis (Gundersen and Bulinski, 1986a). Additional evidence for the polymerization of the Tyr form is that Tyr tubulin is the predominant monomeric species; in untreated cells, we found that Glu tubulin repre-sented only 4.5% of the monomeric tubulin and even this low value is likely to be an overestimate. Two sources could ar-tifactually contribute to an overestimate of the level of Glu monomer: the extraction-induced conversion of Tyr to Glu monomer and the depolymerization of labile microtubules containing Glu tubulin. Although it is clear that Tyr polymer is converted to Glu polymer upon extraction (more Glu tubu-lin was recovered in the polymer fraction than originally ex-isted in the whole cell [Table II]), we do not have evidence that an analogous conversion occurred in the monomer frac-tion. In fact, the lack of detectable Glu tubulin in the mono-mer fraction prepared from nocodazole-treated cells, which contained 95% monomeric tubulin, suggests that conversion of the monomeric tubulin pool did not occur at a significant rate during extraction. The depolymerization of labile microtubules is more likely to contribute to the Glu mono-mer levels that we have determined. Although we did not de-tect a dramatic breakdown of microtubules in extracted cells

by immunofluorescence, the depolymerization of a small percentage of the microtubule polymer would have a significant effect on the Glu tubulin levels in the relatively smaller pool of monomeric tubulin. For example, if one-third of the recovered monomeric tubulin came from the breakdown of polymeric tubulin, which contained ~15% Glu tubulin, then all the Glu tubulin in the monomer pool (4.5%) could be accounted for by microtubule depolymerization.

Because it is difficult to correct for breakdown of labile microtubules during extraction, we feel that a better estimate of the actual level of Glu monomer can be obtained from nocodazole-treated cells, which are virtually devoid of microtubules. In two separate experiments, we detected no Glu monomer in cells treated with nocodazole (see Fig. 10 B); based on our sensitivity of detection of Glu tubulin on immunoblots (20 ng) and the amount of monomeric tubulin loaded on the immunoblots (1.1–1.4 µg), we estimate that Glu tubulin is <2% of the monomeric tubulin.

The second step in our proposed cycle is the postpolymerization conversion of Tyr to Glu tubulin, presumably carried out by the enzyme tubulin carboxypeptidase. Several groups have detected carboxypeptidase activity toward tubulin in brain extracts (Argarana et al., 1978; Kumar and Flavin, 1981; Arce and Barra, 1983; 1985), yet this enzymatic activity had not been reported in material from sources other than brain tissue. The increase in Glu tubulin and the decrease in Tyr tubulin in the extracted cells (Table II) is direct evidence that tubulin carboxypeptidase is active in the TC-7 cells used in this study. Characterization of the partially purified brain enzyme has revealed that the enzyme is highly specific for tubulin (Kumar and Flavin, 1981), that its activity co-purifies with microtubules (Arce and Barra, 1983), and that it prefers polymeric over monomeric tubulin as a substrate (Kumar and Flavin, 1981; Arce and Barra, 1985).

Evidence of postpolymerization modification of Tyr to Glu tubulin in vivo was obtained in two types of experiments. Using three different agents, each with a distinct mechanism of interfering with microtubule depolymerization, we observed an accumulation of Glu tubulin in microtubules. Although azide is likely to have profound consequences for other cellular components, including the inhibition of the ATP-requiring ligase (Raybin and Flavin, 1977a), taxol and vinblastine interact specifically with tubulin and have minimal effects on other cellular processes (Dustin, 1984). Additionally, the taxol and azide experiments demonstrate that all microtubules are potential substrates for the carboxypeptidase, since both treatments led to substantial levels of Glu tubulin in every microtubule. This result is important because it establishes that postpolymerization detyrosination is not limited to a subset of the microtubules. In the second set of experiments we directly observed the appearance of Glu tubulin in microtubules regrown after release from nocodazole, Colcemid, or cold treatments. In each case, the reappearance of Glu microtubules was delayed ~25 min after the reappearance of Tyr microtubules. Analysis of monomer and polymer fractions of cells released from nocodazole showed that the newly polymerized microtubules did not contain detectable Glu tubulin (again, reflecting the preponderance of Tyr tubulin in the monomer pool); only later did Glu tubulin begin to accumulate in microtubules. This analysis additionally demonstrated that microtubules at steady-state are sus-

ceptible to the carboxypeptidase; a plateau in the level of polymeric tubulin was reached by 5–10 min after release from nocodazole, while the accumulation of polymeric Glu tubulin continued for at least 60 min (Fig. 10).

In both the taxol and nocodazole release experiments, we found that Glu tubulin did not appear at measurable levels in the monomer pool (Figs. 9 and 10). This limits the possibility that monomeric Glu tubulin is polymerized at specific times or locations to create a population of Glu microtubules. Additional evidence discounting this possibility is the pattern of Glu recovery we detected by immunofluorescence in cells released from depolymerizing conditions; instead of the sudden reappearance of microtubules stained with Glu antibody, we observed increasing levels of grainy Glu staining in polymer until the stage at which distinct Glu microtubules were detected. The grainy pattern of Glu staining presumably represents increasing detyrosination of Tyr subunits along microtubules. It remains a formal possibility that the Glu tubulin in microtubules is created, in part, by a coupled mechanism in which Tyr tubulin is converted to Glu tubulin as the subunit adds onto the end of a microtubule. However, the simultaneous presence of microtubules with different levels of Glu tubulin, yet with a constant level along their length (Geuens et al., 1986), is more consistent with a postpolymerization modification of Tyr microtubules than with a coupled polymerization-conversion mechanism. It is also clear from taxol treatment of cells that Tyr tubulin can be converted to Glu tubulin in the virtual absence of any monomer (see Fig. 9).

Can the Glu tubulin in microtubules be retyrosinated while residing in polymer? Currently, we do not have direct evidence ruling out this possibility; but it is clear that on balance, the net direction is toward the formation of Glu tubulin. In addition, the ligase has not been found to use polymeric tubulin as a substrate in in vitro assays (Arce et al., 1978). Although the lack of complete detyrosination of taxol-stabilized microtubules or vinblastine paracrystals of tubulin (even after 24 h) may result from inaccessibility of the bundled microtubules to the carboxypeptidase, it could also be due to retyrosination of polymer. In azide treatments in which the ligase is presumably inhibited, nearly complete detyrosination was observed in as little as 4 h.

At this time, we have only limited evidence for the other half of the cycle, namely the breakdown of Glu microtubules and the subsequent retyrosination of the resulting Glu monomer. Nonetheless, these steps can be inferred from existing information. For example, since all interphase microtubules undergo depolymerization at the onset of mitosis (see Dustin, 1984), it is clear that, at least at this time, Glu microtubules would also be broken down. From the rate of formation of Glu microtubules after microtubule regrowth, (~30 min to regenerate an array of Glu microtubules similar to that seen in an interphase cell), one would predict that unless Glu microtubules break down, they should accumulate during interphase. Although there is significant cell-to-cell variability in the number of Glu microtubules in a population of cells (Fig. 1 b; Gundersen et al., 1984), we have not observed dramatic differences in the number of Glu microtubules over the cell cycle (our unpublished observations).

Evidence for the last step in the cycle, the retyrosination of Glu monomer, is also indirect. The lack of significant activity of the ligase toward polymeric tubulin (Arce et al.,

1978) and its ability to form a complex with monomeric tubulin in vitro (Raybin and Flavin, 1977a) predict that this enzyme would be active in retyrosinating monomeric Glu tubulin in vivo. An ATP-dependent tyrosine ligase activity specific for tubulin has been found in extracts of brain and cultured cells, as well as in many other sources (Raybin and Flavin, 1977a; Deanin et al., 1977; Preston et al., 1979, 1981). In addition, we observed no Glu monomer after depolymerization of microtubules (see Fig. 10); since these cells contained detectable Glu tubulin in polymer before depolymerization, this suggests that Glu tubulin is retyrosinated upon entering the monomer pool. Furthermore, we have observed the rapid disappearance of Glu monomer in cells microinjected with Glu tubulin (Webster et al., 1987). Thus, retyrosination of Glu monomer is a normal component of this cycle. Coupled with results above, these data suggest that the breakdown of Glu microtubules and the subsequent retyrosination do contribute to the cycle as depicted in Fig. 11; however, further experimentation will be necessary to completely establish their significance and relationship to the other steps in the cycle.

Can this cycle account for the observed distribution of Tyr and Glu microtubules? As mentioned above, we estimate that a Glu microtubule can be created in as little as 20 min. Given a half-life of microtubules in tissue culture cells of 5–20 min (Saxton et al., 1984; Schulze and Kirschner, 1986), one-eighth to one-half of the microtubules present at any instant would have accumulated enough Glu tubulin to have become a "Glu microtubule." Estimates of the percentage of the total microtubules that are of the Glu type in an average TC-7 cell ($\sim$15–20%; our unpublished observations) are squarely within this range. Since every microtubule is a potential substrate for the carboxypeptidase (see above), the random detyrosination of microtubules, coupled with stochastic breakdown of microtubules and efficient retyrosination of Glu tubulin entering the monomer pool, are sufficient to explain the distribution of Tyr and Glu microtubules we have observed.

What is the significance of such a cycle for tubulin function? In considering the possibilities, it is useful to recall the levels of the various species in vivo: Tyr monomer, $\sim$35% (of total cell tubulin); Tyr polymer, $\sim$55%; Glu polymer, $\sim$10%; and Glu monomer, probably <1% (based on the level determined from nocodazole-treated cells). As discussed above, these levels of Glu monomer and polymer are likely to be overestimates; nonetheless, even if the Glu levels are accurate, it is clear that the cycle is inefficient in generating two species of monomer that might differ in either their ability to polymerize, their degradation rates, or their effect on the autoregulation of tubulin synthesis (Cleveland et al., 1981). In fact, the cycle is efficient in maintaining the monomer pool as a single species, due to the apparently rapid retyrosination of Glu tubulin entering the pool.

If the role of this cycle is not to generate different monomeric species, then the most likely possibility remaining is that it is used to generate polymers with distinct biochemical properties. Although we have not made a detailed analysis, the results of the present study already point to one significant difference between Tyr and Glu microtubules: most or all Tyr microtubules are growing, while many Glu microtubules are not. This conclusion is based on the following considerations. In two previous studies, nearly all cellular microtubules (hence, most of the Tyr microtubules) were found to add microinjected tubulin subunits to their ends distal to the centrosome (Soltys and Borisy, 1985; Schulze and Kirschner, 1986). In the former study, all of the growing microtubules were identified with a Tyr-specific antibody, demonstrating that these were, in fact, Tyr microtubules. Our conclusion that many Glu microtubules are not growing stems from two experimental observations: (a) the existence of Glu microtubules that are stained along their entire length only with the Glu antibody; and (b) our determination that Tyr tubulin is the predominant species in the monomer pool. Any Glu microtubules that are growing would be expected to contain segments at their distal ends that consisted of >98% Tyr tubulin (based on our estimate that <2% of the monomer pool is Glu tubulin).

How can the property of not adding subunits and, perhaps, other properties of Glu microtubules, be translated into functional differences between Glu and Tyr microtubules? The possibilities can be divided into two categories, depending upon the relative stabilities of the Glu and Tyr classes of microtubules. If Glu microtubules are less stable than Tyr microtubules, postpolymerization detyrosination could be involved in the control of microtubule depolymerization. On the other hand, if Glu microtubules are more stable, detyrosination could allow these less dynamic microtubules to perform a "differentiated" function (e.g., struts for maintenance of cell shape, different tracks on which vesicles could move, etc.). As discussed above, the number of Glu microtubules and their rate of formation are consistent with a role for detyrosination in depolymerization; however, at this time we have no evidence that detyrosination is necessary or even that it promotes depolymerization. Evidence that supports a role of detyrosination of microtubules in one of the functions of differentiated microtubules is the finding that the specialized assemblies of microtubules (e.g., axonemes, neurites, marginal bands) in differentiated cells characteristically contain elevated levels of Glu tubulin (Gundersen and Bulinski, 1986b). Also, preliminary studies on interphase microtubules in cultured cells have shown that Glu microtubules are relatively more stable against the action of some microtubule-depolymerizing agents (our unpublished results). In any case, the cycle established in the present study equips cells with a mechanism for generating and maintaining distinct populations of microtubules with biochemically different properties.

References

Arce, C. A., and H. S. Barra. 1983. Association of tubulinyl-tyrosine carboxypeptidase with microtubules. FEBS (Fed. Eur. Biochem. Soc.) Lett. 157:75–78.

Arce, C. A., and H. S. Barra. 1985. Release of C-terminal tyrosine from tubulin and microtubules at steady state. Biochem. J. 226:311–317.

Arce, C. A., M. E. Hallak, J. A. Rodriguez, H. S. Barra, and R. Caputto. 1978.

Capability of tubulin and microtubules to incorporate and to release tyrosine and phenylalanine and the effect of the incorporation of these amino-acids on tubulin assembly. *J. Neurochem.* 31:205–210.

Argarana, C. E., H. S. Barra, and R. Caputto. 1978. Release of [^{14}C]-tyrosine from tubulinyl-[^{14}C]-tyrosine by brain extract. Separation of a carboxy peptidase from tubulin tyrosine ligase. *Mol. Cell Biochem.* 19:17–22.

Barra, H. S., C. A. Arce, and R. Caputto. 1980. Total tubulin and its aminoacylated and nonaminoacylated forms during the development of rat brain. *Eur. J. Biochem.* 109:439–446.

Barra, H. S., C. A. Arce, J. A. Rodriguez, and R. Caputto. 1974. Some common properties of the protein that incorporates tyrosine as a single unit into microtubule proteins. *Biochem. Biophys. Res. Commun.* 60:1384–1390.

Barra, H. S., J. A. Rodriguez, C. A. Arce, and R. Caputto. 1973. A soluble preparation from rat brain that incorporates into its own proteins [^{14}C]-arginine by a ribonuclease-sensitive system and [^{14}C]-tyrosine by a ribonuclease-insensitive system. *J. Neurochem.* 20:97–108.

Bershadsky, A. D., and V. I. Gelfand. 1981. ATP-dependent regulation of cytoplasmic microtubule disassembly. *Proc. Natl. Acad. Sci. USA.* 78:3610–3613.

Caron, J. M., A. L. Jones, and M. W. Kirschner. 1985. Autoregulation of tubulin synthesis in hepatocytes and fibroblasts. *J. Cell Biol.* 101:1763–1772.

Cleveland, D., M. A. Lopata, P. A. Sherline, and M. W. Kirschner. 1981. Unpolymerized tubulin modulates the level of tubulin mRNAs. *Cell.* 25:537–546.

Cumming, R., R. D. Burgoyne, and N. A. Lytton. 1984. Immunocytochemical demonstration of α-tubulin modification during axonal maturation in the cerebellar cortex. *J. Cell Biol.* 98:347–351.

Deanin, G. G., S. F. Preston, and M. W. Gordon. 1981. Carboxyl terminal tyrosine metabolism of alpha tubulin and changes in cell shape: Chinese hamster ovary cells. *Biochem. Biophys. Res. Commun.* 100:1642–1650.

Deanin, G. G., W. C. Thompson, and M. W. Gordon. 1977. Tyrosyltubulin ligase activity in brain, skeletal muscle, and liver of the developing chick. *Dev. Biol.* 57:230–233.

DeBrabander, M., G. Geuens, R. Nuydens, R. Willebrords, and J. DeMey. 1981a. Microtubule assembly in living cells after release from nocodazole block: the effects of metabolic inhibitors, Taxol and pH. *Cell Biol. Int. Rep.* 5:913–920.

DeBrabander, M., G. Geuens, R. Nuydens, R. Willebrords, and J. DeMey. 1981b. Taxol induces the assembly of free microtubules in living cells and blocks the organizing capacity of centrosomes and kinetochores. *Proc. Natl. Acad. Sci. USA.* 78:5608–5612.

Dustin, P. 1984. Microtubules. 2nd edition. Springer-Verlag, New York.

Geiger, P. J., and S. P. Bessman. 1972. Protein determination by Lowry's method in the presence of sulfhydryl reagents. *Anal. Biochem.* 49:467–473.

Geuens, G., G. G. Gundersen, R. Nuydens, F. Cornelissen, J. C. Bulinski, and M. DeBrabander. 1986. Ultrastructural colocalization of tyrosinated and detyrosinated alpha tubulin in interphase and mitotic cells. *J. Cell Biol.* 103:1883–1893.

Gundersen, G. G., and J. C. Bulinski. 1986a. Distribution of tyrosinated and nontyrosinated alpha tubulin during mitosis. *J. Cell Biol.* 102:1118–1126.

Gundersen, G. G., and J. C. Bulinski. 1986b. Microtubule arrays in differentiated cells contain elevated levels of a post-translationally modified form of tubulin. *Eur. J. Cell Biol.* 42:288–294.

Gundersen, G. G., M. H. Kalnoski, and J. C. Bulinski. 1984. Distinct populations of microtubules: tyrosinated and nontyrosinated alpha tubulin are distributed differently *in vivo. Cell.* 38:779–789.

Hiller, G., and K. Weber. 1978. Radioimmunoassay for tubulin: a quantitative comparison of the tubulin content of different established tissue cultured cells and tissues. *Cell.* 14:795–804.

Kilmartin, J. V., B. Wright, and C. Milstein. 1982. Rat monoclonal antitubulin antibodies derived by using a new nonsecreting rat cell line. *J. Cell Biol.* 93:576–582.

Kumar, N., and M. Flavin. 1981. Preferential action of a brain detyrosinolating carboxypeptidase on polymerized tubulin. *J. Biol. Chem.* 256:7678–7686.

Kumar, N., and M. Flavin. 1982. Modulation of some parameters of assembly of microtubules *in vitro* by tyrosination of tubulin. *Eur. J. Biochem.* 128:215–222.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T$_4$. *Nature.* 227:680–685.

Moskalewski, S., J. Thyberg, and U. Friberg. 1980. Cold and metabolic inhibitor effects on cytoplasmic microtubules and the golgi complex in cultured rat epiphyseal chondrocytes. *Cell Tissue Res.* 210:403–415.

Murofushi, H. 1980. Purification and characterization of tubulin-tyrosine ligase from porcine brain. *J. Biochem.* 87:979–984.

Murphy, D. B. 1982. Assembly-disassembly purification and characterization of microtubule protein without glycerol. *Methods Cell Biol.* 24:31–49.

Nath, J., M. Flavin, and J. I. Gallin. 1982. Tubulin tyrosinolation in human polymorphonuclear leukocytes: studies in normal subjects and in patients with the Chediak-Higashi Syndrome. *J. Cell Biol.* 95:519–526.

Nath, J., M. Flavin, and E. Schiffman. 1981. Stimulation of tubulin tyrosinolation in rabbit leukocytes evoked by the chemoattractant formyl-methionyl-leucyl-phenylalanine. *J. Cell Biol.* 91:232–239.

Osborn, M., and K. Weber. 1976. Cytoplasmic microtubules in tissue culture cells appear to grow from an organizing structure towards the plasma membrane. *Proc. Natl. Acad. Sci. USA.* 73:867–871.

Preston, S. F., G. G. Deanin, R. K. Hanson, and M. W. Gordon. 1979. The phylogenetic distribution of tubulin: tyrosine ligase. *J. Mol. Evol.* 13:233–244.

Preston, S. F., G. G. Deanin, R. K. Hanson, and M. W. Gordon. 1981. Tubulin: tyrosine ligase in oocytes and embryos of *Xenopus laevis. Dev. Biol.* 81:36–42.

Raybin, D., and M. Flavin. 1977a. Enzyme which specifically adds tyrosine to the α-chain of tubulin. *Biochemistry.* 16:2189–2194.

Raybin, D., and M. Flavin. 1977b. Modification of tubulin by tyrosylation in cells and extracts and its effect on assembly in vitro. *J. Cell Biol.* 73:492–504.

Rodriguez, J. A., and G. G. Borisy. 1978. Modification of the C-terminus of brain tubulin during development. *Biochem. Biophys. Res. Commun.* 83:579–586.

Rodriguez, J. A., and G. G. Borisy. 1979. Tyrosination state of free tubulin subunits and tubulin disassembled from microtubules of rat brain tissue. *Biochem. Biophys. Res. Commun.* 89:893–899.

Saxton, W. M., D. L. Stemple, R. J. Leslie, E. D. Salmon, M. Zavortink, and J. R. McIntosh. 1984. Tubulin dynamics in cultured mammalian cells. *J. Cell Biol.* 99:2175–2186.

Schiff, P. B., and S. B. Horowitz. 1980. Taxol stabilizes microtubules in mouse fibroblast cells. *Proc. Natl. Acad. Sci. USA.* 77:1561–1565.

Schiff, P. B., J. Fant, and S. B. Horowitz. 1979. Promotion of microtubule assembly *in vitro* by taxol. *Nature (Lond.).* 277:665–667.

Schulze, E., and M. Kirschner. 1986. Microtubule dynamics in interphase cells. *J. Cell Biol.* 102:1020–1031.

Soltys, B. J., and G. G. Borisy. 1985. Polymerization of tubulin *in vivo*: direct evidence for assembly onto microtubule ends and from centrosomes. *J. Cell Biol.* 100:1682–1689.

Thompson, W. C. 1982. The cyclic tyrosination/detyrosination of alpha tubulin. *Methods Cell Biol.* 24:235–255.

Thompson, W. C., G. G. Deanin, and M. W. Gordon. 1979. Intact microtubules are required for rapid turnover of carboxyl terminal tyrosine of alpha tubulin in cell cultures. *Proc. Natl. Acad. Sci. USA.* 76:1318–1322.

Tornqvist, H., and B. Belfrage. 1976. Determination of protein in adipose tissue extracts. *J. Lipid Res.* 17:542–545.

Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350–4354.

Valenzuela, P., W. Quiroga, J. Zaldivar, W. J. Rutter, M. W. Kirshner, and D. W. Cleveland. 1981. Nucleotide and corresponding amino acid sequences encoded by α and β tubulin mRNAs. *Nature (Lond.).* 289:650–655.

Villasante, A., D. Wang, P. Dobner, P. Dolph, S. A. Lewis, and N. J. Cowan. 1986. Six mouse α-tubulin mRNAs encode five distinct isotypes: testis-specific expression of two sister genes. *Mol. Cell. Biol.* 6:2409–2419.

Webster, D. R., G. G. Gundersen, J. C. Bulinski, and G. G. Borisy. 1987. Assembly and turnover of detyrosinated tubulin in vivo. *J. Cell Biol.* 105:265–276.

Wehland, J., M. C. Willingham, and I. V. Sandoval. 1983. A rat monoclonal antibody reacting specifically with the tyrosylated form of α-tubulin. I. Biochemical characterization, effects on microtubule polymerization *in vitro*, and microtubule polymerization and organization *in vivo. J. Cell Biol.* 97:1467–1475.