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Microtubules containing detyrosinated tubulin are less dynamic

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Peptide antibodies specific for tyrosinated (tyr-tubulin) or detyrosinated α-tubulin (glu-tubulin) have been generated for studying the relative stability of microtubules enriched in either form of α-tubulin. Treatment of Vero cells with nocodazole has revealed that interphase microtubules rich in glu-tubulin (glu-microtubules) are resistant to higher concentrations of the microtubule-disrupting drug than the microtubules containing only tyr-tubulin (tyr-microtubules). Glu-tubulin is enriched in centrioles and mid-bodies, but absent from the first interphase microtubules that have repolymerized in late telophase. Tubulin (including both forms) has been labeled with rhodamine (rh-tubulin) and microinjected into Vero cells to study *in vivo* the dynamic properties and incorporation rates of tubulin into microtubules rich in either glu- or tyr-tubulin. Tyr-microtubules are significantly more rapidly labeled by the microinjected rh-tubulin than glu-microtubules. Ten minutes after injection, rh-tubulin is present in virtually all tyr-microtubules. The half-time of turnover of glu-microtubules is ~1 h. Even several hours after microinjection, some of the glu-microtubules have consistently not incorporated visible amounts of rh-tubulin. These results suggest that tyr- and glu-microtubules respectively represent relatively dynamic and stable subclasses of interphase microtubules.

Key words: detyrosinated tubulin/stable microtubules/rhodamine-labeled tubulin/microinjection/peptide antibodies

Introduction

Microtubules are directly involved in several vital processes occurring within the cell. They provide, for example, tracks for translocation of vesicles and organelles, and they play a crucial role in establishing cytoplasmic polarity (for reviews cf. Solomon, 1981; Schliwa, 1984; Allen *et al.*, 1985; Kirschner and Mitchison, 1986; Vale *et al.*, 1986). The different membrane compartments, including the Golgi complex, endosomes and lysosomes are not randomly distributed in the cytoplasm. Instead they seem to be maintained in close proximity to one another in the centrosomal region, probably the functionally optimal position (Thyberg and Moskalewski, 1985; Hopkins, 1986). Tight control of polarity of the cytoplasm is important since cells may have to adapt rapidly and efficiently to changes in their environment. If the microtubules were to perform these specific functions, control of their stability and their asymmetric distribution is required.

The dynamic properties of microtubules have been studied in living cells microinjected with modified microtubule subunits (Keith *et al.*, 1981; Kreis *et al.*, 1982a; Saxton *et al.*, 1984; Scherson *et al.*, 1984; Soltys and Borisy, 1985; Schulze and Kirschner, 1986, 1987). These experiments have suggested that microtubules turnover and grow very rapidly *in vivo*, with most microtubules exchanging with a half-time of ~10 min. However, recently, minor subpopulations of interphase microtubules have been found to be more stable in that they resist exchange for several hours (Webster *et al.*, 1986; Schulze and Kirschner, 1987), or in that they are less sensitive to nocodazole or colchicine (Piperno *et al.*, 1987).

Distinct subclasses of interphase microtubules have recently been described in tissue culture cells. They contain post-translationally modified subunits of tubulin: detyrosinated α-tubulin (Gunderson *et al.*, 1984; Geuens *et al.*, 1986), or acetylated α-tubulin (Piperno *et al.*, 1987). The increased number of glu-microtubules in differentiated cells may suggest stabilization of these microtubules (Gundersen and Bulinski, 1986a). It has also been speculated that differentiation of neighboring microtubules depends on relative differences in age (Kirschner and Mitchison, 1986). Older microtubules, according to that hypothesis, might be exposed longer to the cytoplasmic enzymes involved in post-translational modification of polymeric tubulin.

The goal of this study is to examine whether the subclasses of microtubules that contain predominantly tyrosinated or detyro-

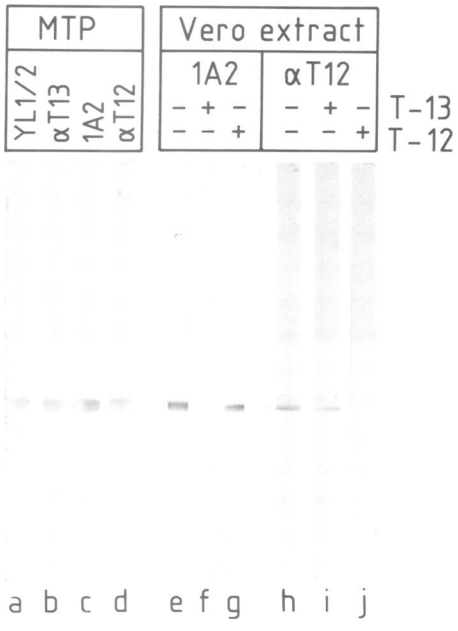

Fig. 1. Characterization of antibodies against tubulin-peptides by immunoblotting. Twice cycled microtubules from bovine brain **(a–d)** and Vero cell extracts **(e–j)** were electrophoresed using SDS–PAGE and transferred onto nitrocellulose paper. The nitrocellulose paper was then incubated with (a) a rat monoclonal antibody against α-tubulin (YL1/2, Kilmartin *et al.*, 1982; obtained from Dr J.Kilmartin, MRC, Cambridge); (b) αT13; (c,e) 1A2; and (d,h) αT12. Binding of 1A2 or αT12 to tubulin was competed with preincubation of the antibodies with excess of the peptides T-13 (f,i) or T-12 (g,j).

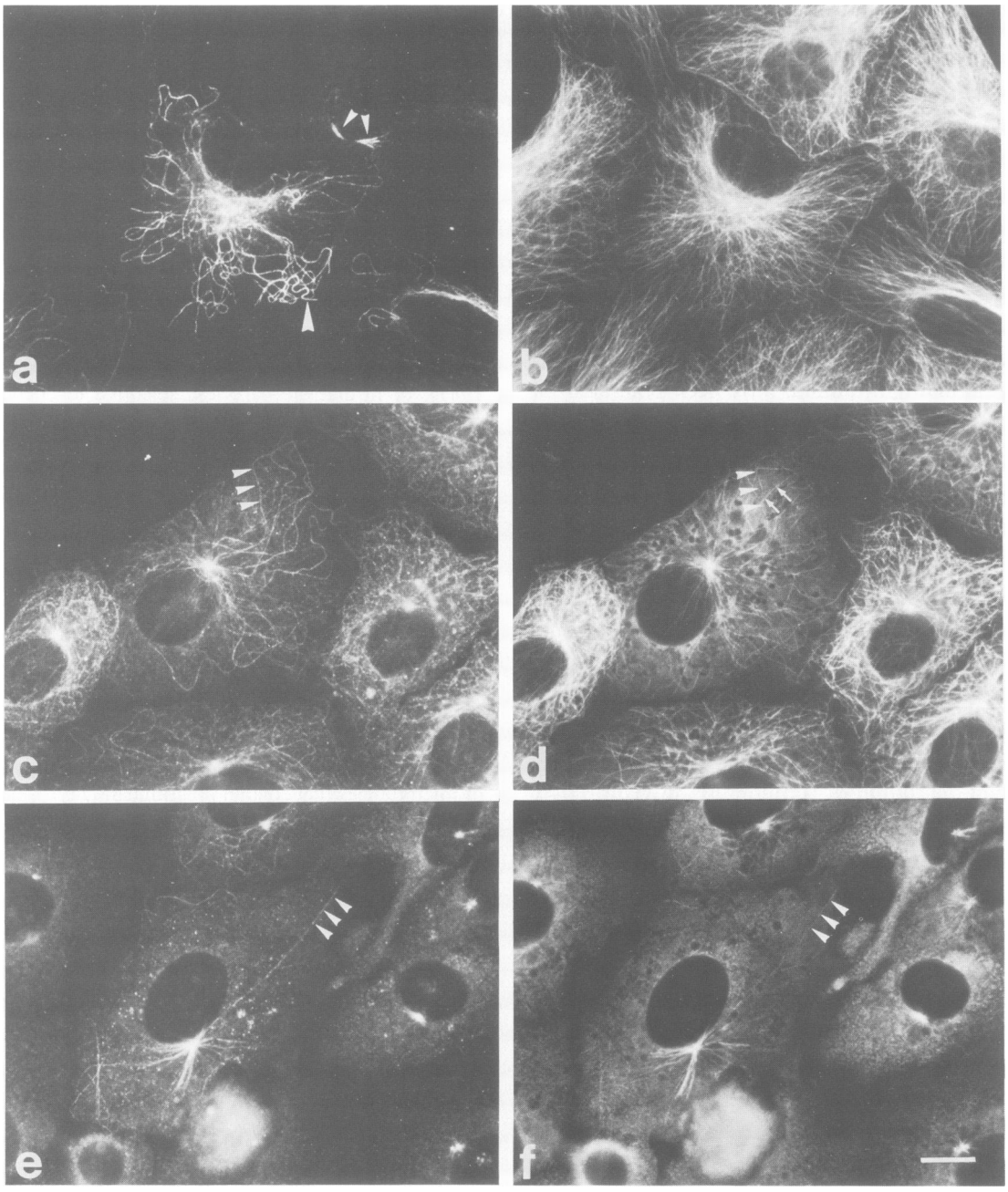

Fig. 2. Glu-microtubules are more resistant to nocodazole than tyr-microtubules. Vero cells were treated with **(a,b)** no, **(c,d)** 0.3 μM, and (e,f) 1 μM nocodazole for 1 h, subsequently fixed according to protocol (1) and double labeled with αGlu (a,c,e) and 1A2 (b,d,f). (a,b), (c,d) and **(e,f)** represent pairs of photographs showing identical cells. The glu-microtubules labeled by αGlu (a) clearly represent a minor subclass of sinuous microtubules apparently different from the dense framework of straight tyr-microtubules (b). The small arrowheads in (a) indicate mid-body microtubules strongly labeled with αGlu. The larger arrowhead in (a) points to a particularly tortuous glu-microtubule at the cell periphery. A striking lack of glu-microtubules is observed in the two daughter cells in the upper right part of (a). The cells treated with the low concentration of nocodazole shown in (c,d) contain fewer microtubules than the non-treated cells. Most of the remaining microtubules now contain both glu- and tyr-tubulin (arrowheads). Occasionally microtubules containing exclusively tyr-tubulin can be detected at the cell periphery (arrows). Treatment of cells with 1 μM nocodazole leaves only a few, centrosome-nucleated microtubules containing both glu- and tyr-tubulin (arrowheads in e,f). Bar = 10 μm.

sinated α-tubulin exhibit different dynamic properties. I have shown that glu-microtubules are more resistant to the microtubule depolymerizing drug nocodazole. Moreover, incorporation of microinjected rh-tubulin into the tyr-microtubules occurs very rapidly, whereas a number of glu-microtubules remain unlabeled by the rh-tubulin for several hours. Thus, two populations of microtubules have been characterized *in vivo*: the very dynamic tyr-microtubules and the more stable glu-microtubules.

Results

Characterization of antibodies against synthetic peptides containing carboxyterminal sequences of α-tubulin

A similar strategy to the one reported by Gundersen *et al.* (1984) was used to obtain antibodies against the tyrosinated and detyrosinated forms of α-tubulin. Polyclonal (αT13) and monoclonal (1A2) antibodies were raised against a synthetic carboxyterminal

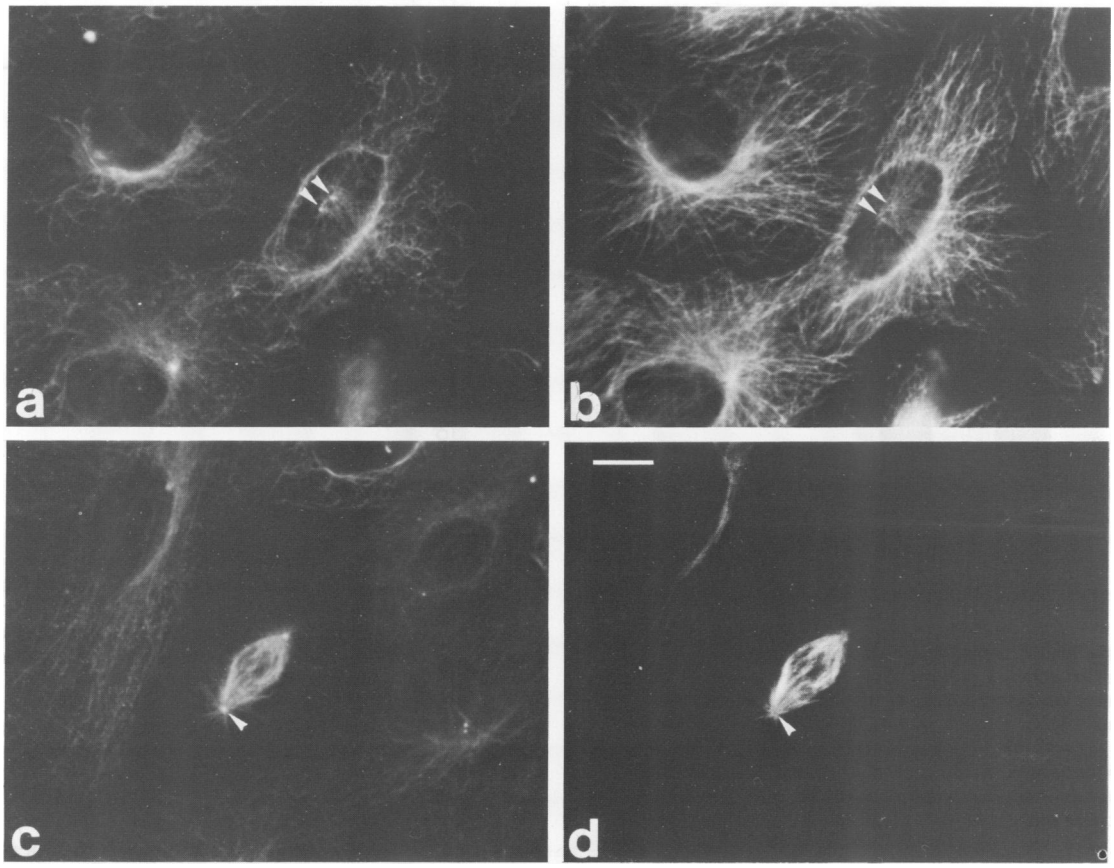

Fig. 3. Glu-tubulin is enriched in centrioles. Vero cells were fixed according to protocol (1) and double labeled with αGlu **(a,c)** and 1A2 **(b,d)**. The cells photographed in (a,b) and (c,d) are identical. Glu-tubulin is strongly enriched in the centrioles of interphase cells (a) or in spidle poles (c). These same structures were not labeled with 1A2, specific for tyr-tubulin (b,d). The fluorescence signal of 1A2-labeling of the cells neighboring the mitotic cell is underexposed because of the strong signal of the mitotic spindle. Centrioles are indicated by arrowheads. Bar = 10 μm.

peptide (T-13) of the tyrosinated form of α-tubulin. Polyclonal antibodies were also generated in rabbits against a synthetic peptide (T-12) containing the carboxyterminus of detyrosinated α-tubulin (αT12). All three antibodies reacted specifically with tubulin from calf brain (Figure 1 b–d), Vero cells (Figure 1 e,h) and all other mammalian cell lines tested so far (including NRK, BHK, MDCK, PtK_2, and AtT-20). 1A2 also reacted with yeast and *Xenopus* tubulin (data not shown).

Competition experiments with the peptides T-12 and T-13 revealed that binding of these antibodies to tubulin on nitrocellulose filters (Figure 1 f,j) and in fixed tissue culture cells (data not shown), was completely abolished when the antibodies were pre-incubated with excess of the respective peptides. Pre-incubation of 1A2 or αT13 with T-12 and αT12 with T-13 had no effect on their binding to tubulin on nitrocellulose filters (Figure 1 g,i) or in fixed tissue culture cells (data not shown). Binding of 1A2 and αT13 was significantly decreased when tubulin was treated with carboxypeptidase A (data not shown). Residual binding was probably due to incomplete removal of carboxyterminal tyrosine by the peptidase. Thus, 1A2 and αT13 (αTyr) react specifically with epitopes present in tyr-tubulin, but not in the carboxyterminal peptide of glu-tubulin. Most likely they recognize the carboxyterminal tyrosine region as does the rat anti-tubulin YL1/2 described by Wehland *et al.* (1983). That pre-incubation with T-13 did not inhibit αT12 (αGlu) binding to tubulin suggests that these antibodies recognize an epitope associated with a specific conformation of glu-tubulin which is different from the conformation of tyr-tubulin. This specific conformation might be an α-helical structure formed by the amino acids 440–450 of glu-tubulin (Ponstingl *et al.*, 1979,1981).

Glu-microtubules are more resistant to nocodazole than tyr-microtubules

The relative resistance of glu- and tyr-microtubules to treatment of cells with the microtubule disrupting drug nocodazole was tested by double immunofluorescence using αGlu and 1A2 (Figure 2). Glu-microtubules were very sinuous in untreated Vero cells (Figure 2a), similar to the glu-microtubules described previously by Gundersen *et al.* (1984) in TC-7 cells and by Geuens *et al.* (1986) in CV-1 and PtK_2 cells. The microtubules which were heavily labeled with αGlu clearly represented a minor subclass of microtubules in interphase cells when compared to the number of the straighter tyr-microtubules labeled with 1A2 (Figure 2b). The number of glu-microtubules could vary in a given population of cells from 0 up to ~100 per cell. Most of the tyr-microtubules were depolymerized when Vero cells were exposed for 1 h to low doses of nocodazole (0.3 μM). Under these conditions few tyr-microtubules remained which were not also immunolabeled with αGlu (Figure 2 c,d). At higher concentrations of nocodazole (1 μM for 1 h) only very few microtubules remained and they contained both glu- and tyr-tubulin (Figure 2 e,f).

Upon removal of nocodazole (10 μM) from Vero cells containing completely disrupted microtubules, the tyr-microtubules

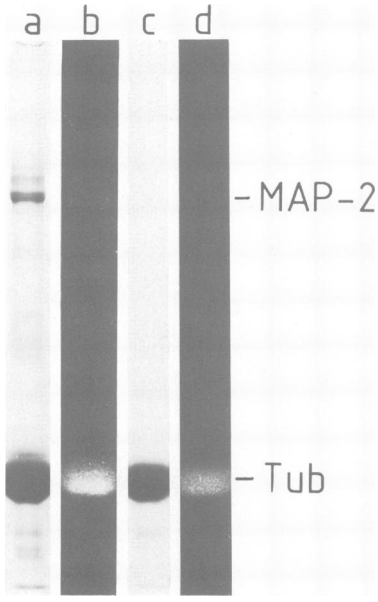

Fig. 4. Characterization of rh-tubulin by SDS gel-electrophoresis. Rhodamine-labeled microtubule proteins, desalted over Sephadex G-50 **(a,b)**, and phosphocellulose purified rh-tubulin **(c,d)** were analyzed by SDS–PAGE using a linear gradient of 4–15% acrylamide. Rhodamine labeled bands on the gel were visualized under u.v. light (b,d), before the gel was stained with coomassie-blue (a,c). The rhodamine labeled bands comigrated with the coomassie-blue stained bands corresponding to the microtubule-associated proteins (e.g. MAP-2 indicated; only faint labeling) and tubulin (Tub). No free rhodamine was present in the rh-tubulin sample.

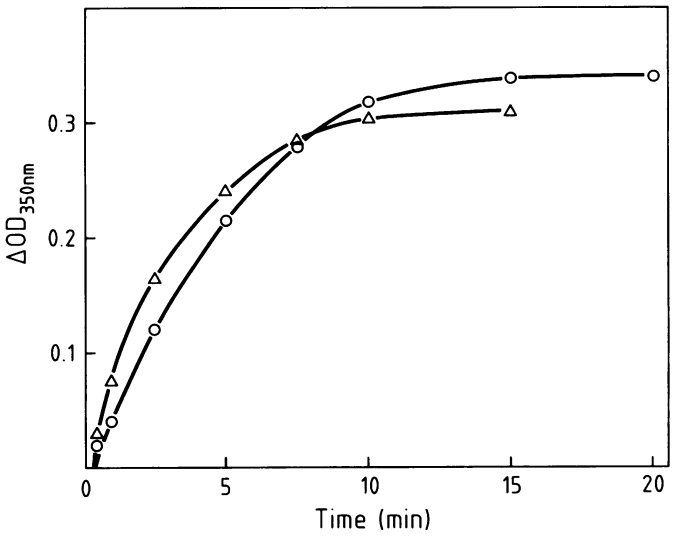

Fig. 5. Kinetics of polymerization of rh-tubulin wih microtubule proteins. Twice-cycled microtubule pellets were depolymerized in PB-buffer on ice and cleared by centrifugation (as described in Materials and methods). To 2.7 mg of the microtubule protein from the supernatant (containing ~35% tubulin) 0.3 mg of rh-tubulin (△–△) or unlabeled phosphocellulose-purified tubulin (O–O) was added. PB-buffer was added to 1 ml and the solution was warmed to 37°C. The polymerization of microtubules at 37°C was induced by the adition of GTP to a final concentration of 1 mM. The increase in turbidity of the solution was measured at different time-points at 350 nm in a Shimadzu UV-190 Spectrophotometer.

reformed first (within 15–30 min), whereas glu-microtubules appeared only ~1 h after the wash-out of the drug (data not shown; similar results were also observed in MDCK cells, Bré *et al.*, 1987). Consistently, none of the interphase microtubules reformed in daughter cells early after mitosis contained detectable amounts of glu-tubulin (Figure 2 a,b).

Mid-body microtubules were enriched in glu-tubulin (small arrowheads in Figure 2a), as were centrioles of interphase Vero cells (arrowheads in Figure 3a) and spindle poles in mitotic cells (arrowheads in Figure 3c). These centrioles could not be stained with 1A2 (arrowheads in Figure 3 b,c) or αTyr (data not shown). A similar distribution of glu-tubulin was observed by Gundersen and Bulinski (1986b) with anti-glu-tubulin antibodies. In contrast to the staining with 1A2 reported here, however, they also oberved centrosomal labeling with the anti-tyr-tubulin antibodies. Both, the mid-body microtubules and particularly the centrioles, were tubulin-containing structures known to persist in cells for long periods of time (cf. for example de Brabander *et al.*, 1986).

Characterization of rhodamine-labeled tubulin

Tubulin (containing both tyr- and glu-tubulin, cf. Figures 1 c,d; 9b) was modified in its polymeric form in the presence of microtubule-associated proteins with rhodamine sulphonychloride. The labeled tubulin was purified by gel filtration chromatography to remove non-covalently bound fluorochrome and chromatographed over phosphocellulose to remove the microtubule-associated proteins (Figure 4). This purified rh-tubulin contained on average ~1 molecule of rhodamine per 4–5 dimers. The polymerization kinetics of microtubule protein (containing ~35% tubulin) with added 10% of the total protein of either rh-tubulin or unlabeled purified tubulin were very similar (Figure 5). The incorporation of rh-tubulin into the microtubules *in vitro* was verified by fluorescence microscopy (Figure 6a). These microtubules containing co-polymerized rh-tubulin were cold-sensitive (Figure 6b). Consistently, ~75% of the added rh-tubulin could be sedimented with the polymerized microtubules by centrifugation and thus co-polymerized with the unlabeled microtubule protein (25% of the input rhodamine remained in the supernatant).

Rh-tubulin was capable of forming centrosome-nucleated microtubule asters similar to those prepared with unmodified tubulin (Figure 6 c,d). Furthermore, microinjected rh-tubulin readily incorporated into the interphase microtubules (Figure 7 a,c for example) and into the microtubules of the mitotic spindle and mid-bodies (data not shown). Therefore, it was concluded that rh-tubulin represents a true marker for the analysis of the dynamic properties of endogenous, cellular tubulin.

Dynamic properties of glu- and tyr-microtubules in vivo

The dynamic properties of interphase microtubules were investigated in living cells by analyzing the rate of incorporation of microinjected rh-tubulin into glu- and tyr-microtubules.

Interphase Vero cells were microinjected with this rh-tubulin and processed for immunofluorescence labeling with antibodies specific for tyr-tubulin (Figure 7) or glu-tubulin (Figure 8). Within 10 min after injection, virtually all tyr-microtubules had incorporated the rh-tubulin (Figure 7 a,b; Table I). Occasionally, early after injection, rh-tubulin was detected exclusively in long segments at the growing ends of interphase microtubules (arrowheads in Figure 7 c,d). This observation is in agreement with previous reports indicating that association of tubulin proceeds only at the growing ends of microtubules (Soltys and Borisy, 1985; Schulze and Kirschner, 1986) and not randomly along their entire length. Rh-labeled microtubules remained visible for at least 24 h (data not shown).

Glu-microtubules incorporated rh-tubulin more slowly than tyr-microtubules. Ten minutes after injection only ~20% of the distinct glu-microtubules which could be resolved at the cell

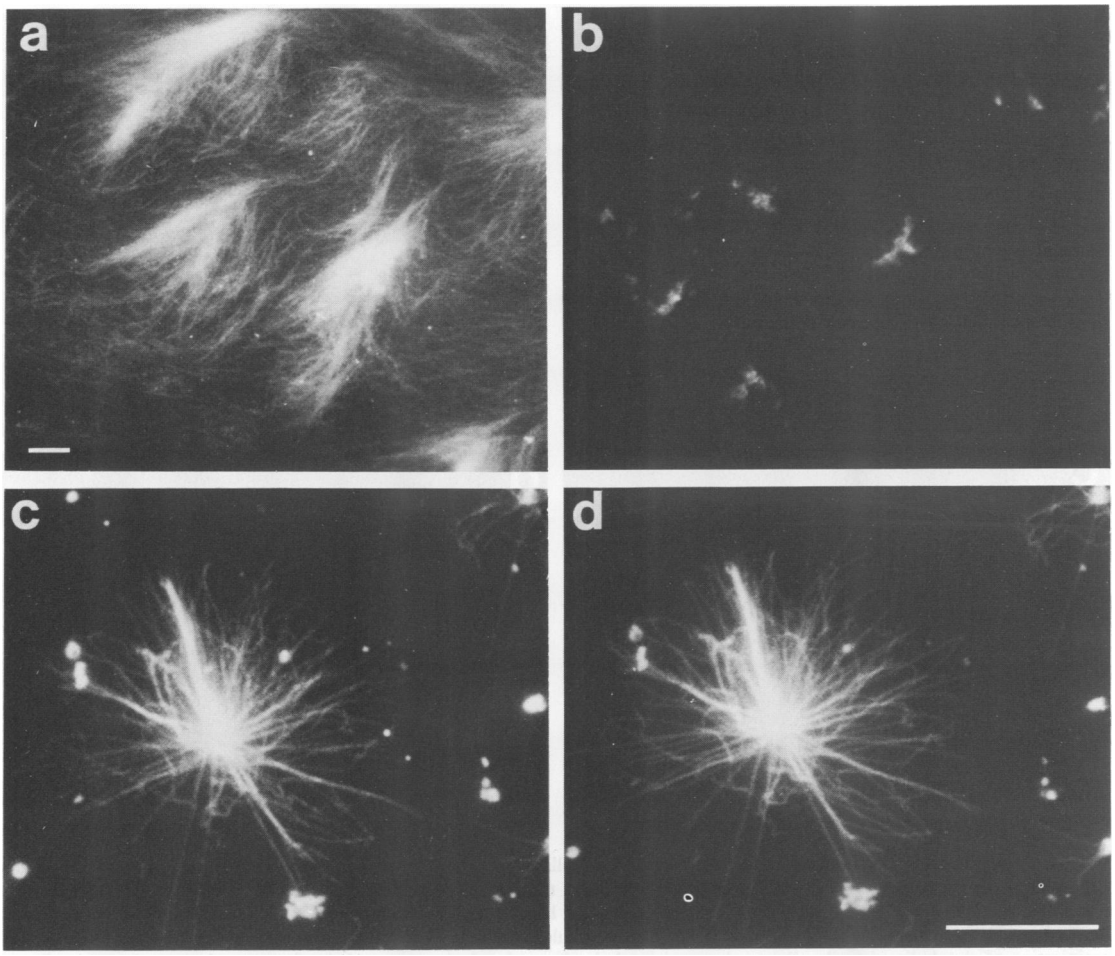

Fig. 6. Incorporation of rh-tubulin into microtubules *in vitro*. Rh-tubulin was copolymerized with microtubule protein (twice cycled) as described in the legend to Figure 5. A 3 μl aliquot of polymerized microtubules was taken 30 min after initiation of polymerization, placed onto a glass slide, and analyzed by fluorescence microscopy. Bundled, as well as probably individual microtubules, were observed which had incorporated the rh-tubulin **(a)**. Virtually all these rhodamine-labeled microtubules depolymerized upon incubation on ice for 30 min **(b)**. Rh-tubulin was also polymerized onto purified centrosomes. The centrosome-nucleated microtubules containing rh-tubulin were fixed onto glass coverslips and labeled with 1A2 and fluorescein-labeled anti-mouse antibodies **(d)**. All the microtubules recognized by the monoclonal antibody were clearly visible by the fluorescence of the rh-tubulin **(c)**. Bars = 10 μm.

periphery contained the fluorescently-tagged marker (the arrowheads in Figure 8 a,b indicate some of the rh-tubulin negative glu-microtubules; Table I). It was assumed in this analysis that the distinct glu-microtubules at the cell periphery had similar kinetic properties to the glu-microtubules in the centrosomal region, densely populated with tyr-microtubules, which could not be resolved and analyzed for rhodamine labeling. One hour after injection of rh-tubulin, ~50% of the population of glu-microtubules was labeled (Figure 8 c,d; Table I). A continuation of rh-tubulin was observed at the growing ends of some glu-microtubules. These, probably newly-polymerized segments, appeared to be devoid of glu-tubulin. Six hours later only a few glu-microtubules could be detected which were not rhodamine-labeled (Figure 8 e,f; Table I). These observations suggested that the half-time of glu-microtubule turnover is ~1 h, ~10 times slower than that for tyr-microtubules.

It was furthermore tested whether rh-tubulin was present in all glu-microtubules which had formed after the microinjection. All glu-microtubules which were analyzed in daughter cells that had been injected before cell division contained rh-tubulin (data not shown). When cells which had been exposed to 10 μM nocodazole to completely depolymerize their microtubules were microinjected with rh-tubulin and returned to normal medium, all the newly formed glu-microtubules, which could be identified at the cell periphery, had incorporated the labeled tubulin (data not shown).

Rh-tubulin-labeled glu-microtubules (including a number of tyr-microtubules) were resistant to low (0.3 μM) concentration of nocodazole (data not shown). To analyze whether new microtubules formed when cells were kept in this low concentration of nocodazole, cells were injected with rh-tubulin, after 1 h of treatment and were kept for a further hour with the same concentration of the drug. These cells exhibited a significant number of rh-labeled tyr-microtubules, but only very few of the glu-microtubules were rhodamine labeled (data not shown).

Tyrosination and detyrosination of microinjected tubulin

Tubulin purified from porcine brain contains predominantly glu-tubulin (Ponstingl *et al.*, 1979). In this study tubulin was prepared from bovine brain microtubules which also contained significant amounts of the detyrosinated form as was shown by immunoblotting (Figure 1d). The fate of this glu-tubulin, after microinjection, was followed in Vero cells that were incubated with 10 μM nocodazole to keep tubulin depolymerized (Figure 9). Considerable labeling with αGlu was observed in cells 5 min after injection of rh-tubulin, but not in the neighboring uninjected cells

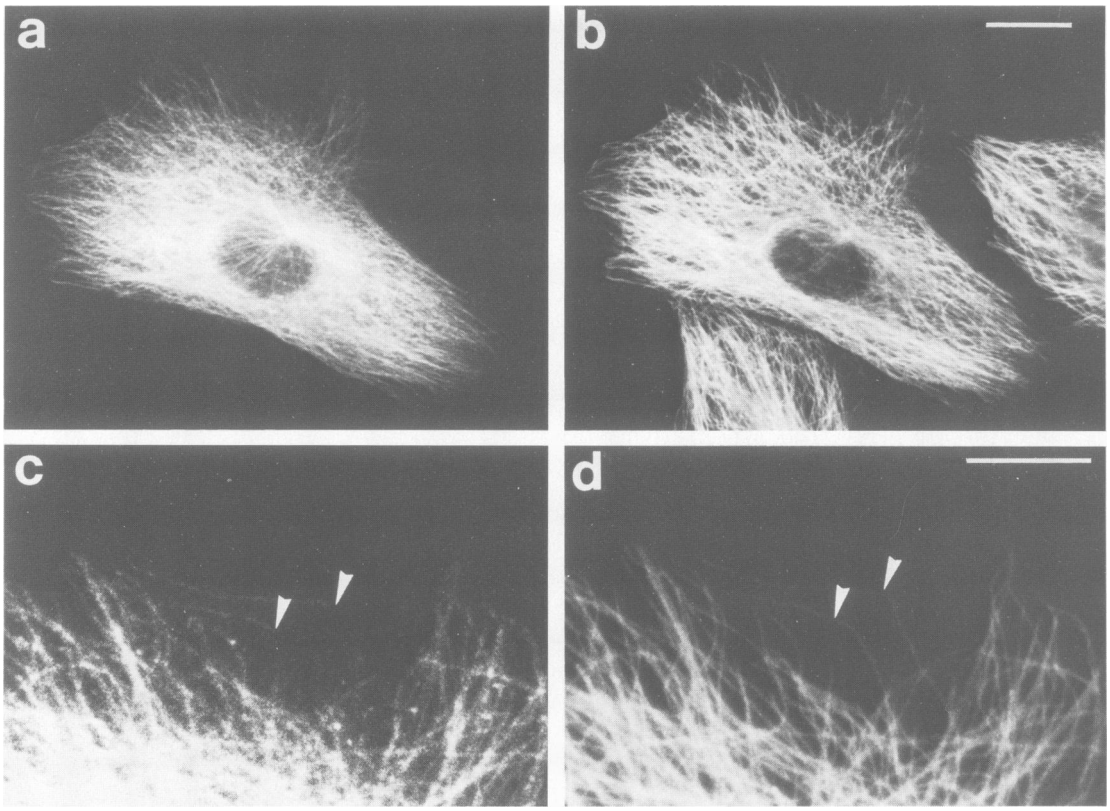

Fig. 7. Incorporation of rh-tubulin into tyr-microtubules in Vero cells. Vero cells were pre-extracted and fixed according to protocol (2) 10 min after microinjection of rh-tubulin. Rh-tubulin which was incorporated into interphase microtubules is shown in **(a,c)**. The overall distribution of tyr-microtubules was visualized by immunolabeling of the same cells with αTyr and fluorescein labeled anti-rabbit antibodies **(b,d)**. (c,d) represent enlargements of the upper area of the cell shown in (a,b) respectively. The arrowheads indicate microtubules containing the rh-tubulin only in a segment extending at their ends. Ten minutes after microinjection of rh-tubulin virtually all interphase microtubules have already incorporated the fluorescently tagged protein. Bars (a,b) = 20 μm; (c,d) = 10 μm.

(Figure 9 a,b). One hour later most of the injected cells were negative for staining with αGlu, indicating that the injected soluble glu-tubulin had been tyrosinated (Figure 9 c,d). Furthermore, the rh-tubulin that was initially incorporated into tyr-microtubules did not bind αGlu (Figure 8). This suggests that the (injected) glu-tubulin is tyrosinated in solution in the cell and that tubulin is detyrosinated in its polymeric form.

Discussion

The majority of the interphase microtubules are highly dynamic structures (for a recent review see Kirschner and Mitchison, 1986), yet, a distinct subpopulation of relatively stable microtubules has been identified by their slower rate of turnover and incorporation of microinjected biotinylated or DTAF-labeled tubulin (Webster *et al.*, 1986; Schulze and Kirschner, 1987). The morphology of these stable microtubules resembles the morphology of the subclass of interphase microtubules containing detyrosinated α-tubulin, the glu-microtubules, characterized extensively in a variety of different cell types (Gundersen *et al.*, 1984, 1986a,b and Geuens *et al.*, 1986). My goal in this study was to assess the dynamic properties of glu- and tyr-microtubules in living cells. The glu- and tyr-microtubules were visualized with specific mono- and polyclonal antibodies and their turnover was determined *in vivo* by microinjection of rhodamine-tagged tubulin.

The analysis *in vitro* of co-polymerization of rh-tubulin with microtubule protein and polymerization of rh-tubulin from isolated centrosomes showed that the fluorescently-tagged protein behaves very similarly to the unmodified tubulin. Furthermore, rh-tubulin incorporated into the microtubules of injected cells with similar kinetics to biotinylated tubulin (Schulze and Kirschner, 1986) or DTAF-labeled tubulin (Soltys and Borisy, 1985). Since rh-tubulin at a concentration of 1.5−2 mg/ml was injected in a volume equivalent to 5−10% of the total cytoplasmic volume (Kreis and Birchmeier, 1982), and since the total tubulin concentration in tissue culture cells is estimated to be ~2 mg/ml (Hiller and Weber, 1978), the increase in cellular tubulin should

Table I. Time course of incorporation of rh-tubulin into glu- and tyr-microtubules

	Time after injection of the rh-tubulin		
	10 min	1 h	6 h
rhodamine labeled tyr-microtubules	98% (225)	ND	ND
rhodamine labeled glu-microtubules	22% (50)	48% (86)	91% (58)

Rh-tubulin was microinjected into Vero cells. Recipient cells were fixed according to protocol (2) 10 min, 1 h, or 6 h afterwards and double labeled with αGlu or αTyr. Individual microtubules at the cell periphery (10−20 cells) were analyzed for presence or absence of incorporated rh-tubulin. Only those immunolabeled microtubules were scored which could also be analyzed unambiguously for rh-tubulin labeling. The percentage of glu- or tyr-microtubules labeled by the injected rh-tubulin was calculated from the total number of microtubules scored (number given in parentheses). ND, not determined.

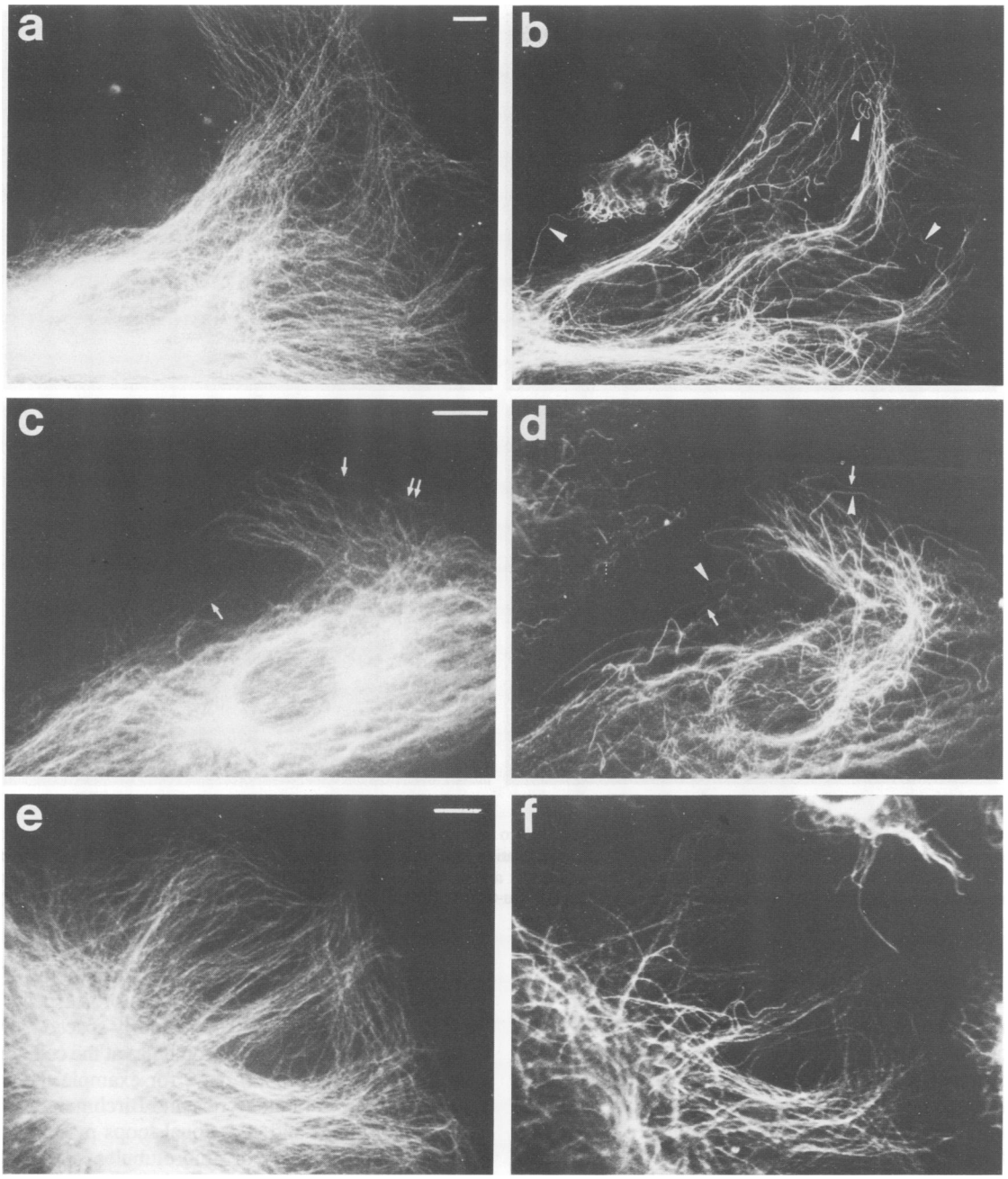

Fig. 8. Incorporation of rh-tubulin into glu-microtubules in Vero cells. Vero cells were pre-extracted and fixed according to protocol (2) 10 min **(a,b)**, 1 h **(c,d)** and 6 h **(e,f)** after microinjection of rh-tubulin. Rh-tubulin incorporated in interphase microtubules is shown in (a,c,e). The overall distribution of the glu-microtubules was visualized by immunolabeling of the same cells with αGlu and fluorescein-labeled anti-rabbit antibodies (b,d,f). The arrows in (c,d) indicate glu-microtubules containing incorporated rh-tubulin. The double-arrow (c) points to a rh-tubulin labeled segment extending the end of a glu-microtubule. Arrowheads indicate glu-microtubules lacking incorporated rh-tubulin. Bars = 10 μm.

remain <10% (for a similar analysis see also Schulze and Kirschner, 1986). Since 20–25% of the injected molecules are modified with rhodamine, up to 2.5% of the tubulin of the recipient cells is labeled. These amounts of microinjected rh-tubulin yielded rapidly a distinct labeling of the cellular microtubules (cf. Figure 7 for example). In comparable experiments using microinjected rhodamine-tagged actin, 1% fluorescently-modified protein yielded sharp and easily visible labeling of the endogenous microfilament system (Kreis *et al.*, 1979, 1982b). In conclusion, rh-tubulin enters the endogenous pool of tubulin and thus it can be used as a marker for the direct analysis *in vivo* of the dynamic properties of the cellular protein.

Microinjected rh-tubulin polymerized *in vivo* into interphase microtubules at their growing ends (Figure 7c) as reported previously (Soltys and Borisy, 1985; Schulze and Kirschner, 1986) and not by random insertion into pre-existing microtubules along their entire length. Virtually all the tyr-microtubules exhibited rhodamine labeling within 10 min after the injection of rh-tubulin. This corresponds well with previously reported half-times of interphase microtubules of 5–15 min (Saxton *et al.*,

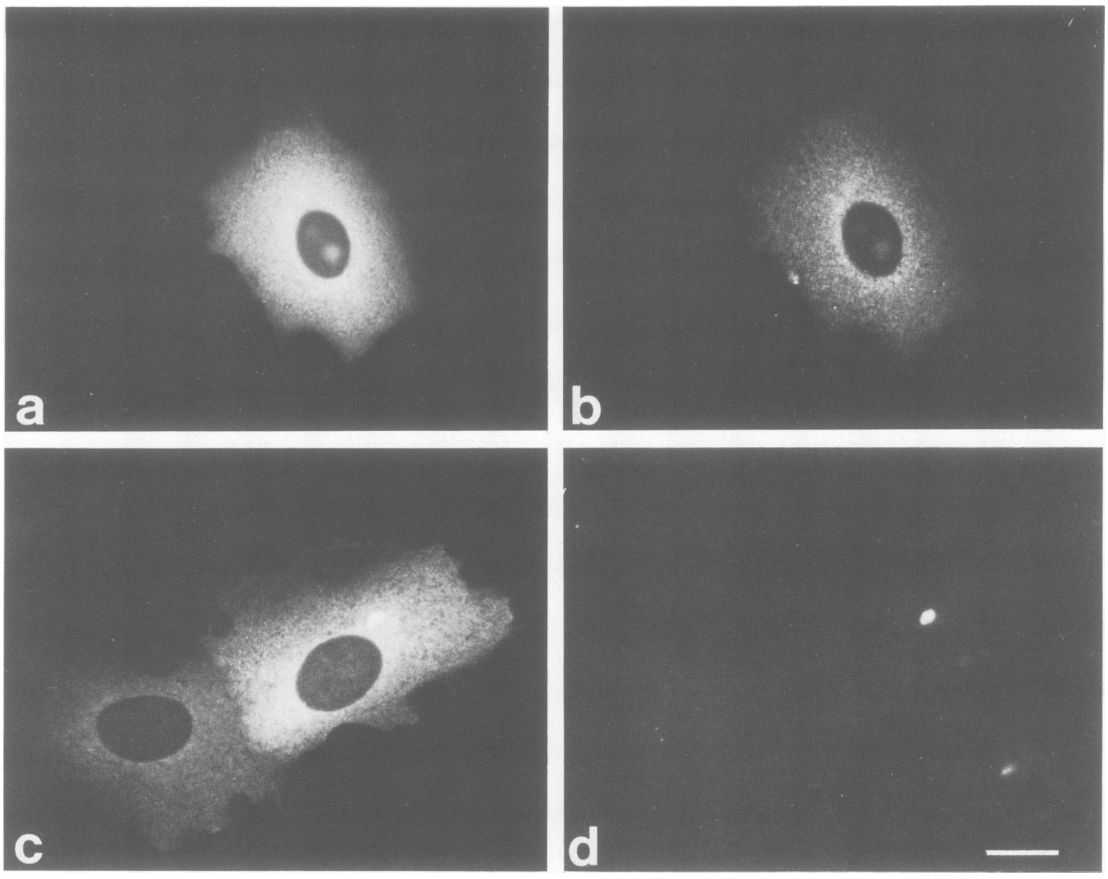

Fig. 9. Tyrosination of microinjected rh-labeled glu-tubulin in Vero cells. Vero cells were treated with 10 μM nocodazole for 1 h before microinjection of rh-tubulin. Injected cells were fixed according to protocol (1) after further incubation in culture medium containig 10 μM nocodazole for 5 min **(a,b)** or 1 h **(c,d)**. Glu-tubulin was visualized with αGlu and fluorescein-labeled anti-rabbit antibodies α(b,d). The distribution of rh-tubulin is shown in (a,c). Virtually all of the injected rh-tubulin, which originally contained significant amounts of glu-tubulin (b), was tyrosinated by the recipient cells within 1 h (d). Bar = 20 μm.

1984; Soltys and Borisy, 1985; Schulze and Kirschner, 1986). Incorporation of rh-tubulin into glu-microtubules, however, was ~10-fold slower than into tyr-microtubules (Table I). A half-time of 1 h for glu-microtubule turnover was estimated by following the disappearance of rh-tubulin negative glu-microtubules with time. Not all of the glu-microtubules could be analyzed for incorporation of injected rh-tubulin because the density of rh-labeled microtubules was too high towards the center of the cells. The half-time for turnover of glu-microtubules was, therefore, measured predominantly in microtubules located at the cell periphery and it is assumed that they share similar dynamic properties with the glu-microtubules in the cell center. The class of stable microtubules observed recently in BSC-1 cells by Schulze and Kirschner (1987) shares similar kinetic properties with the glu-microtubules which are described in this report. Furthermore, glu-microtubules were more resistant to disassembly induced by low concentrations of nocodazole (Figure 2). If one assumes that binding of nocodazole to tubulin subunits prevents addition of this complex to microtubules, then it should be the microtubules with the lowest turnover rates which disappear last (cf. Schulze and Kirschner, 1987, for different results). In conclusion, these results suggest that the glu-microtubules belong to a class of stabilized interphase microtubules, whereas the tyr-microtubules are highly dynamic.

The average age of glu-microtubules is considerably greater than that of tyr-microtubules, as a consequence of the reduced turnover rate. The very sinuous morphology of many of the glu-microtubules may reflect, therefore, aspects of motile activities in the cytoplasm. The regularly spaced hairpin bends frequently observed on glu-microtubules at the cell periphery (larger arrowhead in Figure 2a) may, for example, be caused by contraction of stress fibers (Kreis and Birchmeier, 1980). The high number of bends and occasional loops may also have been induced by movement of microtubules along a more stable cytoplasmic scaffold (other cyotskeletal structures or intracellular membranes). Kinesin, a microtubule-binding protein belonging to an anterograde translocator unit, in fact, is capable of moving microtubules along a substrate *in vitro* (Vale *et al.*, 1985). The physiological role of glu-microtubules in cells is still unknown. Further experiments are required to reveal whether they perform specific functions in the maintenance of cellular or cytoplasmic polarity, or whether they support specific transport functions.

The carboxyterminal tyrosine is encoded by the messenger RNA of α-tubulin (Valenzuela *et al.*, 1981). Therefore, two enzymatic activities must be present in the cytoplasm: one for the removal of the tyrosine by a tubulin tyrosine carboxypeptidase (Hallak *et al.*, 1977), and one for retyrosination by a tubulin tyrosine ligase (Raybin and Flavin, 1977). Both reactions are thought to be reversible. Several lines of evidence suggest that all interphase microtubules are initially tyrosinated. Daughter cells, early after mitosis, usually have only tyrosinated microtubules (Figure 2a) and only rarely can one detect glu-micro-

tubules. The first microtubules that repolymerize after nocodazole wash-out are also exclusively tyr-microtubules. Glu-microtubules can have terminal segments containing only tyr-tubulin (Figure 8c; Bré *et al.*, 1987; cf. however, Geuens *et al.*, 1986, for opposite result). Moreover, glu-tubulin is efficiently retyrosinated in the soluble form in the cytoplasm (Figure 9). In conclusion, these observations indicate that it is tyr-tubulin which polymerizes to form the interphase microtubules, and that detyrosination occurs later on polymeric tubulin.

Are glu-microtubules less dynamic (or more stable) because they have been detyrosinated, or is a stabilized subclass of tyr-microtubules detyrosinated because they grow older and thus remain longer exposed to the enzymatic activity of tyrosinases? So far, it is not known whether and how detyrosination of α-tubulin leads to an increase in stability of interphase microtubules. The delayed appearance of rh-tubulin in glu-microtubules may be explained by assuming that they are predominantly in the shrinking phase, since they may be the oldest ones. If this was the case, then the rate of appearance of rh-tubulin in glu-microtubules would indicate the rate of detyrosination of tyr-microtubules. Yet, the polymerization of tyr-tubulin at the ends of glu-microtubules suggests that microtubules rich in glu-tubulin have not necessarily lost the property of growing. Alternatively, detyrosination of tubulin may lead to a conformational change in the carboxyterminal region of α-tubulin (cf. for example Ponstingl *et al.*, 1979), which may cause glu-tubulin to interact differently with microtubule-stabilizing factors (microtubule-associated proteins, or other cytoskeletal or membraneous components). Both forms of carboxytermini are indeed exposed to the cytoplasm, since they are accessible to microinjected antibodies (Wehland *et al.*, 1983; T.E.Kreis, unpublished results). Since enzymes that detyrosinate tubulin are commercially available (carboxypeptidase A), and since tubulin-tyrosine ligase can be purified from brain tissue (Murofushi, 1980; Schröder *et al.*, 1985), it should be possible to generate pure tyr- or glu-microtubules. Their stability could then be tested *in vitro* (cf. Mitchison and Kirschner, 1984a,b). Furthermore, a specific interaction between these subclasses of microtubules and microtubule-associated proteins could be investigated (see also Kumar and Flavin, 1982).

Obviously, direct visualization *in vivo* of biotinylated tubulin is impossible (Schulze and Kirschner, 1986), and visualization of tubulin derivatized with fluorescein is hampered because it yields too low fluorescence signals and bleaching proceeds far more rapidly than with rh-tubulin (Soltys and Borisy, 1985). The rh-tubulin is, therefore, a much more suitable probe for monitoring microtubules in living cells. Video-enhanced fluorescence microscopy techniques should allow continuous recording of rh-tubulin labeled microtubules, and thus, direct visualization of the dynamic properties of microtubules *in vivo*.

Materials and methods

Cell culture and drug treatment of cells

Vero cells were grown as described (Kreis, 1986b). The partial or complete depolymerization of microtubules was achieved by incubation of Vero cells in culture medium at 37°C containing appropriate concentrations of nocodazole (Calbiochem-Behring, La Jolla, CA, USA).

Antibodies against synthetic peptides

Synthetic peptides containing the eleven carboxyterminal amino acids (440–450) of α-tubulin from porcine brain (Ponstingl *et al.*, 1981) plus an additional aminoterminal lysine on position 439 (T-12), or T-12 with an additional tyrosine at the carboxyterminus (T-13), were prepared by R.Frank and H.Gausepohl (EMBL, Heidelberg). The aminoterminal lysine was introduced to improve efficiency of crosslinking of the peptides to keyhole limpet hemocyanin (Calbiochem-Behring) by glutaraldehyde. The crosslinking of peptides to keyhole limpet hemocyanin and the production of poly- and monoclonal antibodies was carried out as described (Kreis, 1986b). Polyclonal rabbit antibodies against T-12 (αT12, αGlu) and T-13 (αT13, αTyr) were affinity purified on a matrix containing the respective peptides coupled to CNBr-activated sepharose 4B-CL (Pharmacia, Uppsala, Sweden). These antibodies were then incubated with the Sepharose beads containing the other peptide to eliminate any crossreaction with the other form of α-tubulin. A murine monoclonal antibody (IgG-3) was obtained that reacted exclusively with the tyrosinated form of α-tubulin (1A2). Immunoblotting was carried out according to the protocol of Burnette (1981) as described elsewhere (Kreis, 1986b).

Preparation of rhodamine-labeled tubulin

Beef brain microtubules were prepared according to Shelanski *et al.* (1973) using 4 M glycerol and 1 mM GTP in the polymerization buffer (PB: 100 mM Pipes–KOH, 1 mM EGTA, 1 mM $MgCl_2$, pH 6.8). Frozen pellets of twice-cycled microtubules were disassembled in PB. Remaining microtubules were sedimented after 30 min at 0°C at 180 000 *g* for 30 min at 4°C. Five millilitres of the supernatant (5 mg protein/ml) was incubated with 2.5 ml of 12 M glycerol and 3 mM GTP in PB at 37°C to induce polymerization of microtubules. After 15 min, 2.5 ml of PB containing 1 mM GTP and 450 μM rhodamine sulfonylchloride was added (from a 15–25 mM stock solution in acetone, prepared according to Brandtzaeg, 1973). After a further 15 min at 37°C, rhodamine-labeling was stopped with 50 μl of 100 mM glycine pH 8.5 and the microtubules were sedimented at 180 000 *g* for 30 min at 30°C. The pellet was rinsed twice with PB and disassembled at 0°C in 1 ml PB containing 1 mM GTP. Microtubules which had not been disassembled after 30 min were sedimented at 180 000 *g* for 30 min at 4°C. Non-covalently bound rhodamine was removed by gel filtration of the supernatant over a column of 15 ml Sephadex G-50 (Pharmacia, Uppsala) equilibrated with CB-buffer (50 mM Pipes-KOH, 1 mM EGTA, 0.2 mM $MgCl_2$, pH 6.8). The peak fraction of labeled protein was applied to a 3 ml-phosphocellulose column (Whatman P11) equilibrated with CB-buffer. Unlabeled phosphocellulose-purified tubulin was prepared as described (Mitchison and Kirschner, 1984a). The peak of the labeled protein in the flow through was pooled and frozen in aliquots in liquid nitrogen. Polyacrylamide gel-electrophoresis (SDS–PAGE) according to Laemmli (1970) revealed that these peak fractions contained pure, rhodamine-labeled tubulin (rh-tubulin). Protein concentrations were determined by the method of Bradford (1976), using BSA as a standard. The concentration of rhodamine bound to tubulin was determined as described for microfilament-associated proteins (Kreis, 1986a). Following this labeling procedure we obtained rh-tubulin at 1.5–2 mg/ml with fluorochrome to protein (tubulin dimer) ratios of 0.2–0.25.

Centrosome nucleated microtubule asters were prepared as described (Mitchison and Kirschner, 1984a) with rh-tubulin (1.5–2 mg/ml) or unlabeled phosphocellulose-purified tubulin (1.5–2 mg/ml) and purified centrosomes. Glutaraldehyde-fixed microtubule asters were sedimented onto round glass coverslips and post-fixed for 5 min in methanol at −20°C. Immunofluorescence with these microtubules polymerized *in vitro* was performed as described below.

Microinjection

Microinjection of rh-tubulin (1.5–2 mg/ml) into cells grown on glass coverslips was performed as described (Kreis and Birchmeier, 1982; Kreis, 1986b).

Immunofluorescence

Two protocols were used to visualize microtubules in Vero cells: (1) Cells grown on glass coverslips were fixed and extracted by sequential immersions for 4 min in methanol and acetone at −20°C. Afterwards, cells were rinsed with PBS, (2) cells which were microinjected with rh-tubulin were rinsed briefly with PBS and then pre-extracted by dipping the coverslips four times for 3 s into four different beakers, each containing microtubule stabilizing buffer (80 mM Pipes–KOH, 5 mM EGTA, 1 mM $MgCl_2$, pH 6.8) and 0.5% Triton X-100. This treatment reduced the amounts of soluble rh-tubulin and yielded sharper contrast of the rhodamine-labeled microtubules above background. The cells were then fixed as described in the first protocol. No other significant difference with respect to the display of microtubules was observed when the two fixation protocols were compared. Immunolabeling with specific first antibodies and fluorescently labeled second antibodies was then carried out as described (Kreis, 1986b). Fluorescence microscopy and photography was performed using a Zeiss Photoscope III equipped with Leitz objectives (NPL Fluotar 40/1.30 OIL or PL APO 63/1.40 OEL). Photographs were taken onto Kodak Tri-X negative film which was developed at 1600 ASA.

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