Distribution of Tyrosinated and Nontyrosinated α-Tubulin during Mitosis

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Abstract. The C-terminus of α-tubulin undergoes a reversible posttranslational tyrosination/detyrosination. The distributions of the tyrosinated (Tyr) and nontyrosinated (Glu) species during mitosis of cultured cells have been investigated by immunofluorescence using antibodies directed against the C-terminus of either Tyr or Glu tubulin. The distribution of Tyr tubulin differed from that of Glu tubulin at each stage of mitosis; in general, the distribution of Tyr tubulin was similar to that of total tubulin, whereas Glu tubulin had a more restricted distribution. The Glu species was found in half-spindle fibers but was not detected in astral fibers at any stage and was seen in the interzone only during telophase. These results were confirmed by a direct comparison of the distributions of Tyr and Glu tubulin in cells double-labeled with the two antibodies. Evidence for the occurrence of Tyr and Glu tubulin in each class of half-spindle fibers (kinetochore and polar) was obtained from the staining patterns of the two antibodies in cold-treated cells. Immunoblots of extracts prepared from synchronous mitotic cells showed that Glu tubulin was a minor species of the total tubulin in the spindle; no changes in the amount of either Tyr or Glu tubulin were detected at any stage of mitosis. These results show that Tyr tubulin is the major species in the mitotic spindle and is found in all classes of spindle fibers, whereas Glu tubulin is present in small amounts and shows a more restricted distribution. The presence of two biochemically distinct forms of alpha-tubulin in the spindle may be important for spindle function.

THE mechanism of chromosome alignment and subsequent separation during cell division has long fascinated cell biologists. In eukaryotes, a complex structure, the mitotic spindle, is elaborated to effect this chromosome segregation. Studies of the ultrastructure of the mitotic spindle, the dynamics of the spindle during mitosis in living cells, and the effects of various drugs or physical agents on spindle organization have characterized the changes in organization that the microtubules, the constituent elements of the spindle, undergo during mitosis (these topics are subjects of recent reviews: see references 17, 25, 38, 42, and 46). These studies have also clearly established a role for microtubules in chromosome segregation, and coupled with knowledge of the in vitro properties of tubulin (reviewed in references 39 and 47), have led to several models for mitosis (4, 24, 34, 35). Nonetheless, all of these models avoid one of the critical questions raised by studies of the rearrangement of microtubules during mitosis; namely, what is the molecular nature of the control that permits some microtubules to shorten (e.g., kinetochore microtubules during anaphase), while at the same time and virtually in the same place, other microtubules are growing (e.g., interzonal microtubules during anaphase). Explanations based on local changes in the intracellular milieu, such that microtubule polymerization or depolymerization is favored, implicate specialized zones around each spindle microtubule; such mechanisms are hard to envision, much less test.

One possibility that might explain the differences in behavior of individual spindle microtubules is that their component proteins are different. This is essentially the "multi-tubulin hypothesis" (18) applied to the mitotic spindle, although currently there is no evidence for different tubulins within the spindle. Nonetheless, considerable evidence exists for extensive tubulin heterogeneity in the brain (16, 19, 20) and even within a single neuron (21). Similarly, differences at the molecular level have been described between the α-tubulin incorporated into sea urchin flagella and cilia and that present in the cytoplasm (53). This later study provided an even more striking result: the A- and B-subfibers of the axoneme contained different species of tubulin. Thus, there is evidence from some systems that tubulin heterogeneity exists not only within a single cell but within a single organelle.

One possible source for this tubulin heterogeneity is that there are different genes that specify different tubulin proteins (isoforms). Studies of the genetic organization of tubulin have shown that there are at least four different isoforms of both α- and β-tubulin in both vertebrates and invertebrates (11, 44). Tubulin genes have been shown to be expressed in a developmental and tissue-specific manner, although in general more than one isoform is expressed in each tissue. Mutations in the testis-specific β-tubulin of *Drosophila* lead to abnormal meiosis and impared axonemal formation, which suggests that a single isoform can perform multiple microtubule functions (26, 27). For those cases in which multiple

isoforms are expressed, it is not known whether the different isoforms have distinct localizations or functions.

Although genomic complexity can provide for some heterogeneity, it clearly cannot account for all of it. In the brain, as many as 18 isoelectric variants of tubulin have been detected (16). Posttranslational modifications are likely to be the source of this additional heterogeneity. Several posttranslational modifications of tubulin have been described, which include phosphorylation of the β-subunit (15), and acetylation (33) and tyrosination (5, 6) of the α-subunit. Of these three, tyrosination is unique to tubulin. It is a reversible reaction and results in the addition (or removal) of a tyrosine residue from the C-terminus of α-tubulin (2). The reaction occurs in both vertebrates (43) and invertebrates (28) and is thus a good candidate for a modification involved in a ubiquitous microtubule function, such as mitosis.

An aid to our understanding of the function of tubulin posttranslational modifications would be a determination of the intracellular location of the modified and unmodified species. In an earlier report, we described the preparation of peptide antibodies specific for the C-termini of tyrosinated (Tyr)[1] and nontyrosinated (Glu) α-tubulin and found that Tyr and Glu tubulin are largely segregated into different interphase microtubules (22). In this paper, we describe the distribution of these species of α-tubulin during mitosis.

Materials and Methods

Cells

The TC-7 (African green monkey) and PtK_1 (rat kangaroo) cells used in this study were cultured at 37°C, in a humid atmosphere with 5% CO_2, in the following media supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT): Dulbecco's modified Eagle medium (TC-7) or F-10 (PtK_1). Media were obtained from GIBCO (Grand Island, NY). For immunofluorescence studies, cells were seeded onto glass coverslips and fixed 2–3 d later.

Antibodies

The preparation and characterization of the rabbit antibodies to the C-termini of Tyr and Glu α-tubulin have been previously described (22). Fluorescein (FITC) and tetramethyl-rhodamine (RITC) conjugates of goat anti-rabbit IgG were obtained from Cooper Biomedical Inc. (Malvern, PA). Horseradish peroxidase-conjugated goat anti-rabbit IgG was from Miles Laboratories (Naperville, IL).

Immunofluorescence

Cells on glass coverslips were fixed with methanol and rehydrated as previously described (22). Cold treatment of PtK_1 cells was done by removing the growth medium, adding fresh medium chilled in an ice bath, and then placing the dish of cells on a water-ice bath for 30–60 min before fixation. In some experiments, cells were treated with pancreatic carboxypeptidase A (CPA) after methanol fixation (22). Single indirect immunofluorescence with either the Tyr or Glu antibodies and indirect-direct immunofluorescence with the Glu antibody and an RITC-labeled preparation of Tyr-IgG were performed as previously described (22). The Glu and Tyr antibodies were used at 1:50 dilutions of clarified antisera. In this study, we chose Glu and Tyr antisera which showed approximately equal reactivity toward purified brain tubulin, an ~50:50 mixture of Tyr and Glu tubulin (45, 48), in an enzyme-linked immunosorbent assay. Stained preparations were mounted in 10 mM Tris-buffered saline, pH 8.0, with 0.1% NaN_3 and sealed with fingernail polish. Observation was with a Zeiss 63×, 1.4 numerical aperture planapochromat objective (Carl Zeiss, Inc., Thornwood, NY) on a Nikon Optiphot microscope (Nikon Inc., Garden City, NY) equipped with filter cubes B_2 and G for fluorescein and rhodamine fluorescence, respectively. Photographs were taken with Plus-X or Tri-X film using exposure times of 0.5–4 s for indirect immunofluorescence and 4–16 s for direct immunofluorescence using RITC-Tyr IgG. To avoid the slight bleed-through of RITC-fluorescence into the FITC channel, in double-labeled preparations we routinely photographed the RITC-fluorescent image first and then bleached the remaining fluorescence until only a dim image was apparent (~5–10 min). The FITC-fluorescent image was then photographed.

Quantification of Glu and Tyr Tubulin in Synchronized Mitotic Cells

Synchronous TC-7 cells were prepared according to Zieve et al. (58). Briefly, nearly confluent cultures were treated with thymidine (5 mM for 16 h), released from thymidine for 8 h, and then mitotic cells were accumulated by adding nocodazole (0.04 μg/ml) for 3–4 h. Cultures were preshaken just before the addition of fresh medium that contained the nocodazole to remove any loosely adhering cells. For each experiment, we collected the accumulated mitotic cells (still in the presence of nocodazole) by shake-off from eight flasks (75 cm^2). These cells were then washed twice in medium without serum or nocodazole (t_0 = time of addition of second wash), divided into equal aliquots, and cultured at 37°C. At intervals, aliquots were washed rapidly one time with 10 mM Tris-buffered saline, pH 7.4; the cell pellets were then dissolved in SDS sample buffer (2% SDS, 100 mM Tris, pH 6.8, 10 mM dithiothreitol, 20% glycerol, 2 mM phenylmethylsulfonyl fluoride) and boiled 5 min. Cells released from nocodazole at the same time and plated onto polylysine-coated coverslips were fixed at the same time the SDS samples were prepared to assess morphologically the stage of mitosis. Using this protocol, more than 95% of the cells were at prometaphase at t_0, and ~90% of the cells completed mitosis by t_{60} (data not shown).

Immunoblotting was performed to obtain a semiquantitative estimate of the amount of Glu and Tyr tubulin. Samples were electrophoresed on 7.5% SDS polyacrylamide gels (30), transferred to nitrocellulose, and stained with antibodies according to Towbin et al. (54), except that 4-chloro-1-naphthol was used as the chromagen. Glu and Tyr antibodies were used at 1/5,000 dilution of antisera. Samples of synchronous mitotic cells were compared with samples prepared from exponentially growing cells (interphase cells) and from DEAE-purified porcine brain tubulin (41). In control experiments, extracts of interphase cells were treated with CPA before adding SDS and immunoblotting (22).

Results

We have previously described the preparation and characterization of antibodies against Tyr and Glu α-tubulin (22). Antibodies were prepared in rabbits against short peptides corresponding to the C-termini of Tyr and Glu tubulin. Although these peptides differed by only a single amino acid, each antiserum was specific for the corresponding form of α-tubulin, i.e., one recognized only Tyr α-tubulin, while the other recognized only Glu α-tubulin. We have now used these antibodies to determine the distribution of Tyr and Glu tubulin in the mitotic spindle of cultured monkey kidney (TC-7) and rat kangaroo (PtK_1) cells.

Distribution of Tyr and Glu Tubulin during Mitosis

Changes in the pattern of Tyr and Glu tubulin during mitosis were examined in exponentially growing TC-7 cells that were fixed and then stained by indirect immunofluorescence. Representative images of the staining observed with the two antibodies at different stages of mitosis are shown in Fig. 1. Tyr staining was first observed as a small aster at prophase (Fig. 1 *a*), and later appeared as a bipolar spindle in prometaphase (Fig. 1 *c*) and metaphase (Fig. 1 *e*). Tyr staining of the half-spindle remained intense during anaphase (Fig. 1 *g*) and telophase (Fig. 1 *i*), and with the appearance of discernible interzonal fibers at anaphase and telophase, was also observed between the separating chromosomes (Fig. 1, *g* and *i*). At the end of mitosis, the cytoplasmic microtubules of the two daughter cells and the midbody connecting them showed staining with the Tyr antibody (Fig. 1 *k*). One feature common

[1] *Abbreviations used in this paper:* CPA, carboxypeptidase A; FITC, fluorescein; Glu, nontyrosinated α-tubulin; MAP, microtubule-associated protein; Tyr, tyrosinated α-tubulin; RITC, tetramethylrhodamine.

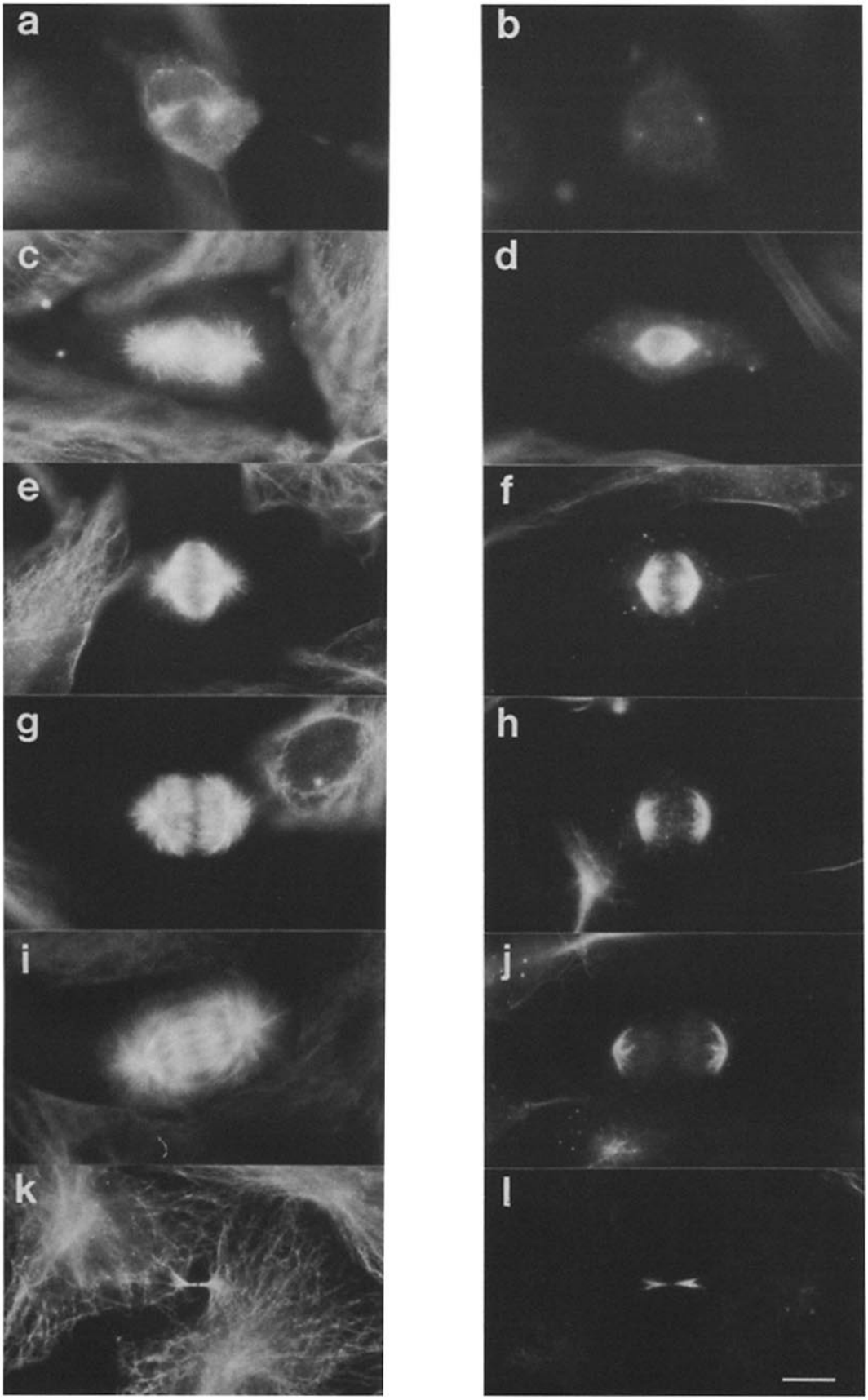

Figure 1. Indirect immunofluorescence of Tyr and Glu Tubulin during mitosis of TC-7 cells. Methanol-fixed TC-7 cells were stained with Tyr (left side; *a, c, e, g, i,* and *k*) or Glu (right side; *b, d, f, h, j,* and *l*) antibodies followed by RITC-goat anti-rabbit IgG and then photographed using epifluorescence illumination. (*a* and *b*) Prophase cells; (*c* and *d*) prometaphase cells; (*e* and *f*) metaphase cells; (*g* and *h*) anaphase cells, note the lack of staining in *h* of the interzone (region on either side of the equator); (*i* and *j*) telophase cells, note the faint staining in *j* of the interzone; (*k* and *l*) daughter cells with a persistent midbody. Bar, 10 μm.

to the different mitotic stages was the bright staining of the astral microtubules with Tyr antibody (Fig. 1, *a, c, e, g,* and *i*). This was in direct contrast to the Glu antibody, which did not stain the astral fibers at any stage of mitosis, including early prophase cells in which the forming spindle is little more than two small asters (Fig. 1, *b, d, f, h,* and *j*). However, like the Tyr antibody, the Glu antibody did stain the half-spindle in mitotic cells that had progressed beyond early prophase (for example, see the cells in prometaphase and metaphase, Fig. 1, *d* and *f*). A second difference in the distribution of Glu and Tyr tubulin was observed in anaphase cells; although the half-spindle remained strongly stained with the Glu antibody, no staining was observed in the interzonal region (Fig. 1 *h*). This difference was more apparent in cells that had progressed to telophase; Glu staining of the half-spindle remained intense, while the interzone of the same cell showed only faint staining (Fig. 1 *j*). By comparison, the staining of telophase cells with the Tyr antibody showed bright labeling of both the half-spindle and the interzone (Fig. 1 *i*). Cells in later stages of telophase showed increased staining with the Glu antibody in the interzonal region, so that after cytokinesis a remnant of the interzonal fibers, the midbody, was strongly labeled (Fig. 1 *l*). A final difference between the distributions of Glu and Tyr tubulin was noted in recently divided daughter cells, distinguishable by the presence of a persistent midbody; the newly formed cytoplasmic microtubules were brightly labeled with the Tyr antibody, while none were detectably labeled with the Glu antibody (compare Fig. 1, *k* and *l*). In summary, Tyr tubulin was found in every class of microtubules in the mitotic spindle, whereas Glu tubulin showed a more restricted distribution, being undetectable in astral fibers, early interzonal fibers, and newly formed cytoplasmic microtubules in daughter cells. We have observed similar Glu and Tyr distributions in mitotic spindles of many other cell types, including rat kangaroo PtK_1 (see Fig. 3), human primary fibroblasts (356 strain), human HeLa, mouse 3T3 and L, SV-40 transformed TC-7, and Chinese hamster ovary, as well as in dividing cells from rat brain and rabbit trachea explants (data not shown). Although spindles from some cell lines (e.g., L and HeLa) showed relatively less intense Glu staining, the similarities in the staining patterns suggest that the distributions of Glu and Tyr tubulin shown in Fig. 1 are common features of all vertebrate spindles.

Several controls indicated that the staining with the Tyr and Glu antibodies was specific. Preimmune serum from each rabbit did not stain either cytoplasmic or spindle microtubules (data not shown). As described previously, preincubation of the antibodies with the same peptide used in the immunogen completely inhibited microtubule staining (22). As a final control, we treated fixed cells with CPA before the immunofluorescent staining. CPA digestion removes the C-terminal tyrosine residue, but leaves the remainder of the α-tubulin chain intact (22, 45). After CPA treatment, no Tyr antibody staining was observed in cells at any stage of mitosis (a metaphase and a telophase cell are shown in Fig. 2, *a* and *c*, respectively). On the other hand, prior CPA treatment dramatically increased the staining with the Glu antibody (Fig. 2, *b* and *d*). It is especially noteworthy that the CPA-digested cells showed bright astral and interzonal staining with the Glu antibody, since these fibers were not stained in untreated cells.

A question that arises in the immunolocalization of two different species is whether the observed pattern reflects the distribution of the two species or is an artifact that results from the differences in the effective concentration of the two antibodies used for localization. In the present study, this is particularly relevant to the distribution of Glu tubulin, since some spindle tubules were not detectably labeled by the Glu antibody. Three pieces of evidence argue that the staining pattern we have observed by immunofluorescence reflects the distribution of Glu tubulin and is not an artifact generated by low Glu antibody concentration. In comparisons of the reactivity of the two antibodies against brain tubulin, which has a Glu/Tyr tubulin composition of ~50/50 (45, 48), the antibodies showed equal reactivity by an enzyme-linked immunosorbent assay (data not shown) and by immunoblots (see Fig. 5). This alone demonstrates that the two antibodies do not have radically different effective concentrations. However, to bias the detection of Glu tubulin in the spindle we have elevated the concentration of Glu antibody used for staining and decreased that of the Tyr antibody. The staining shown in Fig. 1 was obtained with the same dilution (1/50) of each of the two antibodies and similar photographic exposure times (generally, the exposure for Glu staining was twice that for Tyr staining). When the concentration of the Glu antibody used for staining was increased 10-fold (to a 1/5 dilution), the overall Glu staining was much more intense than that observed with the Tyr antibody, yet no staining of astral or anaphase interzonal fibers was observed. Conversely, if the

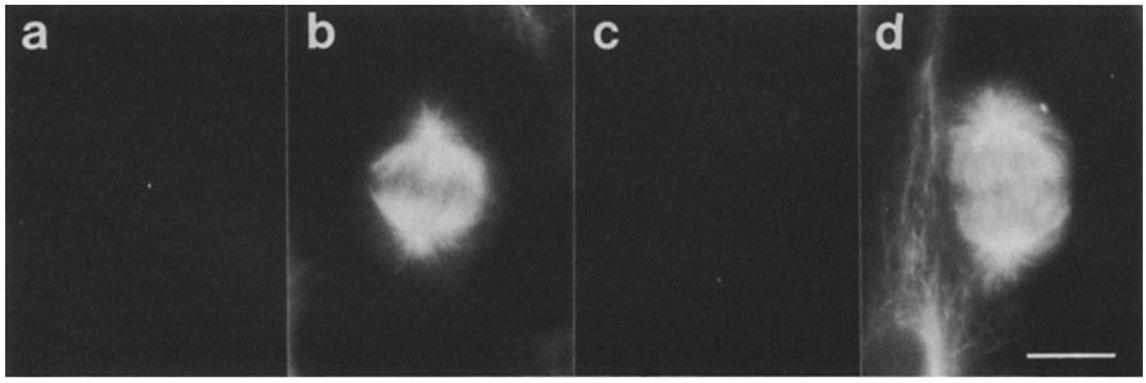

Figure 2. Indirect immunofluorescence of CPA-treated TC-7 mitotic cells. Methanol-fixed cells were treated with CPA and then stained indirectly with Tyr (*a* and *c*) and Glu (*b* and *d*) antibodies. (*a* and *b*) Fluorescence images of CPA-treated metaphase cells; (*c* and *d*) fluorescence images of CPA-treated telophase cells. Bar, 10 μm.

concentration of the Tyr antibody used for staining was decreased eightfold (to a 1/400 dilution), the Glu staining was again more intense than the Tyr staining, yet the Tyr antibody still detectably labeled astral, interzonal, and half-spindle fibers (data not shown). Thus, even when an 80-fold difference in the dilutions of the two antisera was used for staining, we still observed the same patterns as were observed with equal antisera dilutions. Finally, the staining of CPA-treated cells with the Glu antibody also supports the idea that the Glu antibody is not limiting; when additional sites are created by CPA digestion, the Glu staining is increased without changing the concentration of Glu antibody used for staining (see Fig. 2).

Direct Comparison of Glu and Tyr Tubulin Distribution in Mitotic Spindles

The above experiment clearly showed that Glu tubulin was detectable in only a subset of spindle microtubules, whereas Tyr tubulin showed a more widespread distribution. Experiments in which cells were double-labeled with either the Tyr or Glu antibody and a monoclonal antibody to β-tubulin, which detected all spindle microtubules, confirmed that Glu tubulin was restricted in distribution, whereas Tyr tubulin was found in all classes of spindle microtubules (data not shown). To compare the distribution of Glu and Tyr tubulin in individual spindles, we performed double-labeling experiments using an indirect-direct staining protocol; cells were stained with the Glu antibody followed by an FITC-conjugated second antibody and then an RITC-conjugated IgG fraction of the Tyr antibody. We double-stained both TC-7 and PtK_1 cells and observed no Glu antibody labeling of astral fibers in the same cells that showed bright Tyr antibody labeling of astral fibers (Fig. 3). The astral fibers labeled directly with RITC-conjugated Tyr antibody were less intensely fluorescent than those labeled indirectly with the Tyr antibody and RITC-conjugated second antibody (e.g., compare Fig. 3, *a* and *c* with Fig. 1, *c* and *i*), probably due to the lack of amplification with the direct staining method. A direct comparison of the Glu and Tyr staining of the half-spindle showed that there was overlap in the location of the two forms, although in general Glu staining was less intense at every stage and did not extend all the way to the cell equator as did the Tyr staining (see especially, Fig. 3, *a* and *b*, for TC-7 cells and Fig. 3, *e* and *f* for PtK_1 cells). Finally, interzonal fibers in anaphase and telophase were stained brightly with the Tyr antibody yet showed only weak staining with the Glu

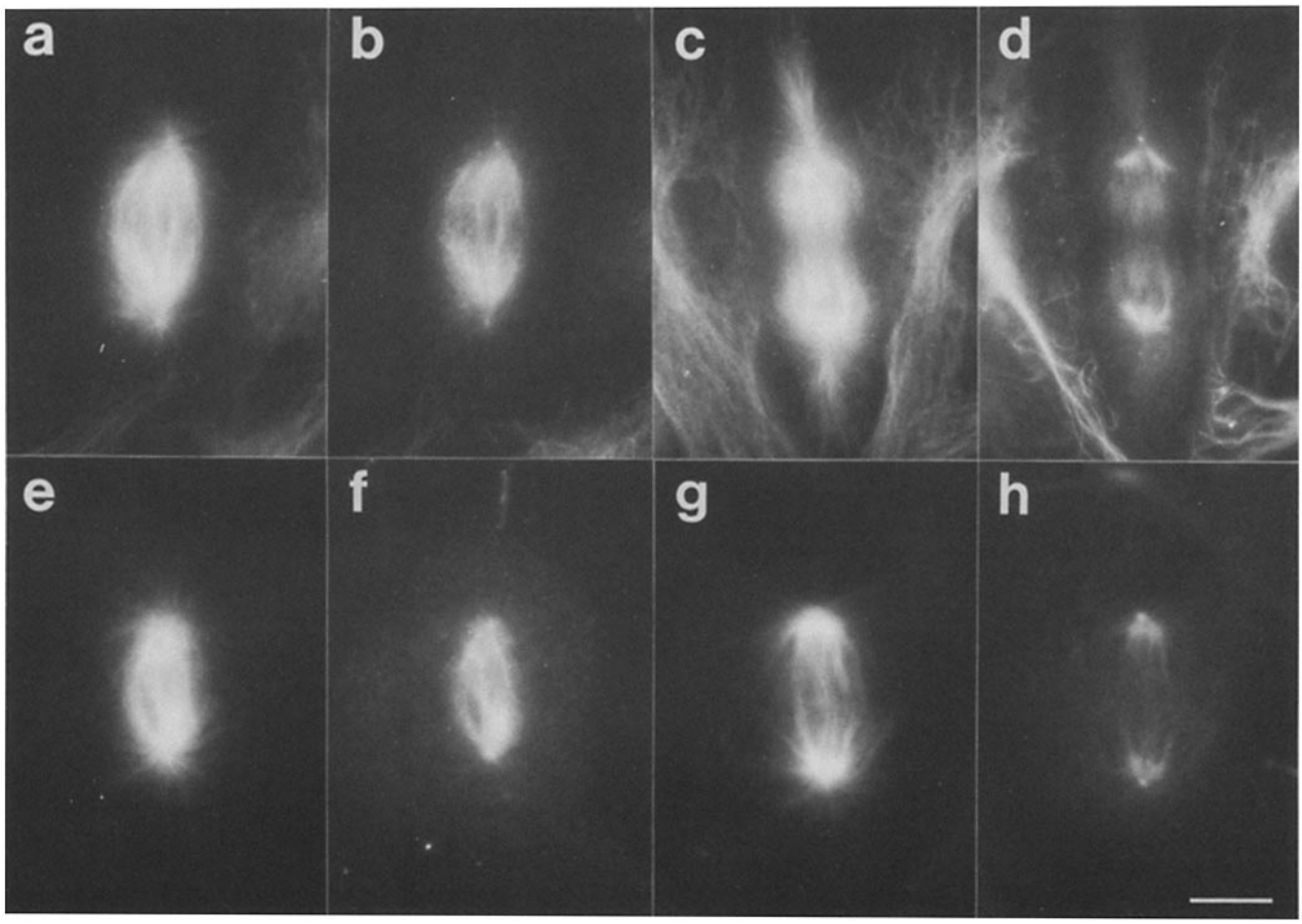

Figure 3. Indirect-direct immunofluorescence of TC-7 and PtK_1 mitotic cells. Methanol-fixed cells were stained indirectly with Glu antibody and FITC-goat anti-rabbit IgG and after a blocking step, were stained directly with RITC-labeled Tyr IgG. Stained cells were photographed under the appropriate illumination for RITC and FITC fluorescence. (*a* and *b*) Prometaphase TC-7 cell, showing Tyr (*a*) and Glu (*b*) tubulin distributions; (*c* and *d*) telophase TC-7 cell showing Tyr (*c*) and Glu (*d*) tubulin distributions; (*e* and *f*) prometaphase PtK_1 cell showing Tyr (*e*) and Glu (*f*) tubulin distributions; (*g* and *h*) anaphase PtK_1 cell showing Tyr (*g*) and Glu (*h*) tubulin distributions. Bar, 10 μm.

antibody (Fig. 3, *c* and *d* show a telophase TC-7 cell; *g* and *h* show an anaphase PtK_1 cell, with just distinguishable interzonal fibers). The decreased Glu staining of fibers near the equator and in the interzone was also observed in comparisons between Glu tubulin and β-tubulin (data not shown).

Distribution of Glu and Tyr Tubulin in Cold-treated Mitotic Cells

The half-spindle is composed of microtubules that are attached to the centrosome (polar microtubules) and to the kinetochore (kinetochore microtubules). Although both Tyr and Glu antibodies stained the half-spindle, it was possible that one of the species was exclusively localized to one of these classes of spindle microtubules. To examine this possibility, we subjected PtK_1 cells to cold (0°C) for 30–60 min and then fixed and stained the cells using the indirect-direct protocol. Under these conditions, only the kinetochore fibers in prometaphase or metaphase cells are stable (9). As shown in Fig. 4, both Tyr and Glu tubulin were detected in the cold-stable kinetochore fibers. Also, note that the level of staining with both antibodies in the cold-treated cells was lower than that observed in untreated cells (compare Fig. 4 with Fig. 3), which suggests that both forms of tubulin were also present in polar microtubules. Similar results were observed in cold-treated prometaphase cells, although the amount of staining with both antibodies was less than that observed in metaphase cells (data not shown). This is consistent with the increase in the number of cold-stable microtubules as cells progress from prophase to metaphase (9).

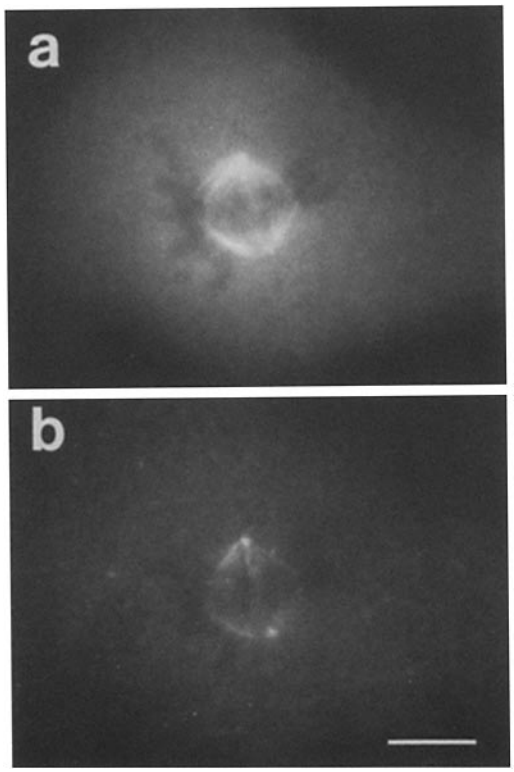

Figure 4. Indirect–direct immunofluorescence of cold-treated PtK_1 mitotic cells. PtK_1 cells were cold-treated, methanol-fixed, and stained by the indirect–direct procedure with Tyr and Glu antibodies (see Fig. 3). (*a* and *b*) Metaphase cell showing Tyr (*a*) and Glu (*b*) tubulin distributions. Bar, 10 μm.

In untreated cells, we observed staining of mitotic centrosomes with the Glu antibody (e.g., Fig. 1 *b*), however, because of the brightness of staining with the Tyr antibody in the vicinity of the centrosome, it was difficult to determine if the centrosome also contained Tyr tubulin. Cold treatment of mitotic cells reduced the number of microtubules near the poles so that staining of the centrosomes with the Tyr antibody, as well as the Glu antibody, became clear (Fig. 4). This was especially evident for later stages of mitosis in which no cold-stable microtubules were found near the centrosomes (data not shown). The centrosomal staining with both antibodies appeared to be specific, since controls, such as preimmune serum or antibodies preincubated with the complementary peptide, showed no centrosomal staining.

Quantification of Glu and Tyr Tubulin in Mitotic Cells

The lack of Glu antibody staining of astral, interzonal, and perhaps some half-spindle fibers, suggested that Tyr was the major form of tubulin in spindle microtubules. However, comparisons of the intensities of immunofluorescent staining can be misleading, especially when the structures that stain undergo reorganization like that observed during mitosis. Therefore, to obtain a semiquantitative estimate of the amount of Glu and Tyr tubulin in the mitotic spindle, we prepared extracts from synchronous cells that had been accumulated at prometaphase with nocodazole and then released for various intervals to capture cells in different stages of mitosis (see Materials and Methods). The levels of Glu and Tyr tubulin were then assessed by immunoblotting. For the TC-7 cells used in this experiment, the cells reached metaphase at ~30 min and completed mitosis at ~60 min after nocodazole release. Substantial amounts of Tyr tubulin were observed in interphase cells (Fig. 5, lane *b*) and throughout mitosis (Fig. 5, lanes *c–h*), while only a very small amount of Glu tubulin was detected in either interphase cells (Fig. 5, lane *j*) or in any of the samples from the mitotic cells (Fig. 5, lanes *k–p*). That this accurately reflects the amount of Tyr and Glu tubulin in the TC-7 cells and is not due to differences in effective antibody concentrations, is shown by the reactivities of the antibodies toward brain tubulin, a roughly equal mixture of Tyr and Glu tubulin (45, 48); at the dilutions used, Tyr and Glu antibodies reacted to about the same extent with purified brain tubulin (Fig. 5, lanes *a* and *i*). No significant differences in the level of Glu (or Tyr) tubulin were detected in any of the samples taken at different intervals after nocodazole release. This was the case even when samples that were highly enriched for one stage were compared, e.g., the sample loaded in lane *m* (and *e*) was composed of 44% late prometaphase and 44% metaphase cells and showed no difference when compared with the sample in lane *o* (and *g*), which was 60% telophase cells and 30% daughter cells with midbodies. Thus, the changes in Glu tubulin distribution we have observed by immunofluorescence do not appear to be due to significant differences in the total amount of Glu tubulin present at the different stages. It is possible that subtle differences would have been detected if samples that contained only one stage were compared, but this level of synchrony was not attainable.

We have previously shown by immunoblotting that the Tyr and Glu antibodies react only with α-tubulin and that CPA digestion can be used to confirm the specificity of the anti-

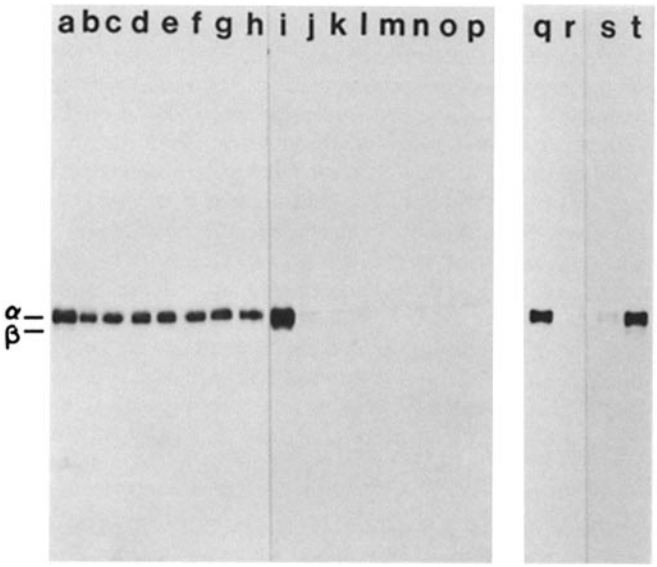

Figure 5. Immunoblot analysis of Tyr and Glu tubulin levels in synchronous mitotic cells. Samples of mitotic cells at different stages were prepared by making extracts of mitotic cells at various intervals after release from a nocodazole block (see Materials and Methods). After SDS-gel electrophoresis and transfer to nitrocellulose, blots were reacted with Tyr (lanes *a–h, q,* and *r*) or Glu (lanes *i–p, s,* and *t*) antibodies and developed with peroxidase. In each case, extract from an equivalent number of cells was loaded on the gel. For comparison, purified brain tubulin (lanes *a* and *i*) and an extract of interphase cells (lanes *b* and *j*) are shown. Lanes *c* and *k*, 0 min after nocodazole release; lanes *d* and *l*, 18 min after nocodazole release; lanes *e* and *m*, 28 min after nocodazole release; lanes *f* and *n*, 36 min after nocodazole release; lanes *g* and *o*, 44 min after nocodazole release; lanes *h* and *p*, 60 min after nocodazole release. The effect of CPA treatment on an extract of interphase cells is shown in lanes *q–t*. Lanes *q* and *r* show Tyr antibody reactivity before and after CPA treatment, respectively; lanes *s* and *t* show Glu antibody reactivity before and after CPA treatment, respectively. The positions of α- and β-tubulin from protein-stained blots of the pure tubulin are shown on the left.

bodies (22). To determine whether the antibodies were specifically reacting in these experiments, we treated extracts of TC-7 interphase cells with CPA before immunoblotting. Without CPA digestion, interphase TC-7 cells had substantial amounts of Tyr tubulin and low levels of Glu tubulin (Fig. 5, lanes *q* and *s*, respectively). CPA digestion of the interphase TC-7 extract completely eliminated Tyr antibody reactivity and dramatically increased Glu antibody reactivity (Fig. 5, lanes *r* and *t*, respectively). In fact, the Glu antibody reactivity of the CPA-digested sample was nearly identical to the Tyr antibody reactivity in the undigested sample (Fig. 5, compare lane *t* with lane *q*). Thus, the low level of Glu tubulin detected in mitotic (or interphase) cell extracts is not an artifact of the immunoblotting.

Discussion

We have carefully examined the distribution of Tyr and Glu α-tubulin during mitosis using peptide antibodies specific for each of the two forms. The specificity of the antibodies has been demonstrated previously (22) and was confirmed in this study by treating either fixed cells or extracted protein with CPA before reaction with the antibodies. In either case, reactivity of the Tyr antibody was completely eliminated by CPA treatment. Glu antibody reactivity was enhanced by CPA treatment, as shown by immunofluorescence detection of certain classes of spindle fibers previously stained only with the Tyr antibody or by an increase in the amount of Glu α-tubulin detected on immunoblots.

Whereas Tyr tubulin was found in all classes of spindle fibers, Glu tubulin showed a more restricted distribution, being undetectable in astral and anaphase interzonal fibers (Figs. 1, 3, and 4). For astral and early interzonal fibers, which only showed Tyr staining, it follows that the individual microtubules that comprise these fibers contain exclusively Tyr tubulin (or at least very low levels of Glu tubulin). A question that arises is, in those cases in which Tyr and Glu tubulin colocalized to a particular class of spindle fibers, e.g., kinetochore fibers, are the individual tubules that comprise the fiber composed of only Glu (or Tyr) tubulin or are the tubules a mixture of the two species? In interphase cells, we have previously observed individual microtubules that were stained predominantly with only one of the two antibodies (22), which suggests that Glu and Tyr tubulin form complementary subsets of interphase microtubules. However, individual microtubules within the spindle of cultured cells, except perhaps astral microtubules, are impossible to resolve with the light microscope. Concerning this question, we did not observe any substantial differences in the distribution of Glu and Tyr tubulin in the half-spindle of either untreated or cold-treated cells double-stained with the two antibodies (see Figs. 3 and 4). This suggests that at least at the level of individual half-spindle fibers, there is no segregation of Glu and Tyr tubulin. We are currently determining whether individual microtubules in the spindle contain one (or both) forms of α-tubulin by immunolocalization at the level of the electron microscope.

Of the two species of α-tubulin in the spindle, Glu tubulin is perhaps the more interesting, since its distribution within the spindle appears to be heterogeneous, i.e., it is detected on only a subset of spindle microtubules. How this heterogeneity is generated is a very interesting question. Recently, we found that Glu microtubules appeared with a constant lag after regrowth of Tyr microtubules in interphase cells after release from nocodazole or Colcemid treatment (manuscript in preparation). This suggests that Glu microtubules are derived from Tyr microtubules, presumably through the action of tubulin carboxypeptidase. Consistent with this idea is the preference of the carboxypeptidase for polymeric tubulin (29). Can such a post-polymerization modification account for the distribution of Glu tubulin we have observed during mitosis? It could if individual classes of spindle microtubules have different half-lives. Longer-lived microtubules then would be expected to have higher levels of Glu tubulin since they would be exposed to the carboxypeptidase longer. Measurements of the bulk flux of tubulin in spindle microtubules, achieved using microinjected, fluorescently labeled tubulin, suggest that microtubule turnover is rapid, on the order of seconds (23, 49, 50); however, the half-life of different classes of spindle microtubules has not yet been determined. When the resolution of these studies is extended, it will be interesting to see if the different spindle microtubules exhibit different turnover rates.

Another very real possibility, one that we cannot completely rule out, is that the heterogeneity in Glu tubulin distribution is more apparent than real. Certainly, the immunofluorescence data shows that the level of Glu tubulin in the metaphase half-spindle and the telophase interzone is higher than

in other places in the cell; but, does this reflect a heterogeneity in Glu tubulin with respect to the distribution of microtubules in the spindle? This is an especially important question because of the differences in microtubule densities within the spindle and the small amount of Glu tubulin in the spindle detected by immunoblotting. For example, we may be able to detect the low level of Glu tubulin in the spindle only when microtubules are bundled together and surpass a critical density. We have attempted to address this problem by increasing the concentration of Glu antibody used for immunofluorescence; yet, even at a 10-fold higher concentration than that which gave bright half-spindle staining, we still observed no Glu staining in the aster or in the anaphase interzone. Also, if stained preparations were viewed with a lower power lens with a greater depth of field, which should sum the contributions from more microtubules, we did not observe any difference in the Glu staining pattern (data not shown). Nonetheless, these approaches may not compensate for certain areas of the spindle with low microtubule densities, such as the astral region. The failure to detect Glu tubulin in the interzone seems harder to discount with this argument. After sister chromatid separation at early anaphase, the number of microtubules in the interzone is about one-half to two-thirds of that in the half-spindle (8, 36, 37). Since the cross-sectional area of the spindle does not change significantly from metaphase to early anaphase, especially if we restrict our consideration to the immediate area on either side of the chromosomes, it is reasonable to assume that the density of microtubules in these two areas is proportional to the microtubule counts. This yields a decrease of maximally one-half in the microtubule density in the early interzone as compared with the half-spindle and would not appear to account for the total lack of Glu staining of the anaphase interzone, since the half-spindle is brightly labeled in the same cell. This leads to two conclusions: only Tyr microtubules contribute to the formation of the interzone, and the increase in Glu staining of the interzone seen at telophase represents an actual increase in the amount of Glu tubulin in these microtubules. Additional factors, such as the bundling of microtubules into stem bodies, which coalesce at late telophase to form the midbody, might also contribute to the increase in Glu staining observed by the time of midbody formation. Immunolocalization at the electron microscopic level should help to distinguish the relative contribution of bundling vs. an actual increase in the content of Glu tubulin in these microtubules.

Regardless of whether there is a heterogeneous distribution of Glu (or Tyr) tubulin in the spindle, the presence of these species shows that each is a subset of the total spindle tubulin. This clearly has ramifications for models of spindle dynamics, since heterogeneity in tubulin could provide the necessary molecular basis for nonuniform behavior exhibited by spindle microtubules. Given the presence of two distinct tubulin species in the spindle, there are many models that could be generated to explain this nonuniform behavior. One possibility is that the two species of α-tubulin interact differentially with microtubule binding proteins. To date, the known microtubule-associated proteins (MAPs), including the 125K and 210K MAPs of primate cells (10), brain tau (12, 13), and brain high molecular weight MAPs (7, 13, 51), show homogeneous distributions in spindles. Similarly, antibodies to four recently identified, putative MAPs from sea urchin eggs uniformly labeled the spindle of sea urchin embryos (55). The existence of a cytoplasmic dynein in the mitotic spindle is still a subject of debate (see reference 3); in those cases in which dynein antibodies were used for immunofluorescence, staining was observed on all classes of spindle microtubules (40). In contrast to all of the above proteins, the distribution of calmodulin in the mitotic spindle appears to be heterogeneous with respect to the distribution of microtubules (1, 14, 56, 57); calmodulin is specifically localized to the half-spindle with the highest apparent concentration near the poles, is undetectable in astral and early interzonal fibers, and is found later in the interzone of telophase spindles. This has been interpreted as showing a high concentration of calmodulin in the half-spindle and a redistribution of calmodulin onto late interzonal fibers. This is very similar, if not identical, to the distribution of Glu tubulin in the spindle, and it is tempting to postulate a specific relationship between the two. However, as discussed above for Glu tubulin, if the calmodulin is directly associated with microtubules, the studies on calmodulin localization may not reflect differences in concentration with respect to microtubules, i.e., calmodulin may be evenly distributed on all spindle microtubules (although see reference 14). With this caveat in mind, it seems equally likely that calmodulin is interacting with Tyr rather than Glu tubulin. Also, we have shown that cold alters the distribution of Glu tubulin in the spindle, while others have observed no effect of cold on the distribution of calmodulin (57). A direct interaction of calmodulin with Glu tubulin also seems unlikely since calmodulin-mediated microtubule depolymerization is thought to result from calmodulin binding to MAPs rather than to tubulin (31, 32, 52).

We have described the distribution of two distinct forms of α-tubulin in the mitotic spindle. The presence of these species in the spindle may provide a biochemical basis for the known heterogeneous behavior of spindle microtubules. However, without a good understanding of the effect of tyrosination/detyrosination on microtubule dynamics, it is premature to speculate on the role this cyclic modification may play in mitosis. Nonetheless, the presence of Glu and Tyr tubulin in the mitotic spindle and the changes that these species undergo during mitosis suggest that tyrosination/detyrosination is intimately involved in mitotic function.

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