Recruitment of antigenic gamma-tubulin during mitosis in animal cells: presence of gamma-tubulin in the mitotic spindle

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SUMMARY

It has been claimed repeatedly that γ-tubulin is exclusively localized at the spindle poles in mitotic animal cells, where it plays a role in microtubule nucleation. In addition to this localization, we have observed a γ-tubulin-specific staining of the mitotic spindle in several animal cells (human, kangaroo rat, mouse, Chinese hamster, Xenopus and Drosophila) using five polyclonal antibodies raised against unique γ-tubulin sequences and four different fixation protocols. In HeLa and PtK2 cells, γ-tubulin was detected in the mitotic spindle from late prometaphase to telophase. In contrast, in other cell types, it was detected in metaphase only. In all cases we failed to detect γ-tubulin in the short aster microtubules at the spindle poles. Electron microscopic observation revealed that at least part of the γ-tubulin localized on the surface of spindle microtubules with a preferential distribution along kinetochore microtubules. In HeLa cells, the amount of antigenic γ-tubulin was fairly constant in the spindle poles during mitosis from prometaphase to telophase. In contrast, γ-tubulin appeared in the mitotic spindles in prometaphase. The amount of γ-tubulin decreased in telophase, where it relocated in the interzone. In metaphase cells about 15-25% of the total fluorescence was localized at the spindle poles, while 75-85% of the fluorescence was distributed over the rest of the spindle. These results suggest that the localization and timing of γ-tubulin during the cell cycle is highly regulated and that its physiological role could be more complex and diverse than initially assumed.

Key words: microtubule, tubulin, gamma-tubulin, mitosis

INTRODUCTION

Microtubules are nucleated on microtubule organizing centers (MTOCs) and exhibit complex dynamic properties involving their interactions with numerous microtubule-associated proteins. They are generated by the non-covalent assembly of α/β-tubulin heterodimers. Recently a new tubulin, called γ-tubulin, has been identified in most eukaryotic cells (Stearns et al., 1991; Zheng et al., 1991). It exhibits about 28-35% identity with both α- and β-tubulins and comparisons of amino acid sequences further suggest that all three tubulins have derived from a common ancestor (Oakley and Oakley, 1989).

The physiological role of γ-tubulin has been approached by gene disruption and microinjection of specific antibodies. Both in Aspergillus and in Schizosaccharomyces, disruption of the gene coding for γ-tubulin causes a reduction in the number and length of cytoplasmic microtubules and the complete absence of mitotic apparatus (Oakley et al., 1990; Horio et al., 1991). In mammalian cells, microinjection of antibodies against γ-tubulin prevents microtubule regrowth during interphase and the assembly of a functional spindle during mitosis (Joshi et al., 1992). Moreover, anaphase cells microinjected with specific antibodies against γ-tubulin failed to normally assemble a midbody (Julian et al., 1993). These observations demonstrate that γ-tubulin is necessary for normal microtubule assembly.

In contrast with all α- and β-tubulins, which are the major constituent of mitotic and interphase microtubules, published localizations in animal cells report that γ-tubulin is restricted to the spindle poles in mitosis and to the centrosome in interphase (Zheng et al., 1991; Stearns et al., 1991). It is located to the spindle plaques both in Aspergillus and Schizosaccharomyces (Oakley et al., 1990; Horio et al., 1991) and in the acentriolar spindle poles in plants (Liu et al., 1993), mouse oocytes (Palacios et al., 1993; Gueth-Hallonet et al., 1993) and in a Drosophila acentriolar cell line (A. Debec and M. Wright, unpublished results). γ-Tubulin has also been detected in other MTOCs that are transiently active during cytokinesis and
located between the two daughter cells. In *Schizosaccharomyces*, it is present in cytoplasmic foci appearing at the cell equator in post-anaphase and most likely plays a role in growth polarity and spatial organization (Horio et al., 1991). In mammalian cells undergoing early cytokinesis, γ-tubulin has been detected in the minus regions of the two interdigitating microtubule arrays that constitute the midbody (Julian et al., 1993). Microtubule polarity determined at the spindle poles, at the interphase centrosomes and in the midbody demonstrates that γ-tubulin is localized at the minus end regions of microtubules (Oakley et al., 1990; Horio et al., 1991; Zheng et al., 1991; Julian et al., 1993).

On the basis of these observations, a simple model for the physiological role of γ-tubulin has been proposed (Oakley et al., 1990; Oakley, 1992) in which it is exclusively localized in the centrosomes and related MTOCs where it plays a role in the morphogenesis of microtubules by an interaction with their minus end region. However, γ-tubulin was initially characterized as an extragenic suppressor of a thermosensitive mutation in *Aspergillus*. The γ-tubulin (Weil et al., 1986; Oakley and Oakley, 1989). It is somewhat surprising that microtubules containing mutant β-tubulin could be destabilized by γ-tubulin located exclusively at their minus ends. For this reason, we have reinvestigated the subcellular localization of γ-tubulin in animal cells by electron microscopy and by fluorescence microscopy using a combination of several affinity-purified, high titer antibodies. Our observations demonstrate that γ-tubulin is not only localized to the spindle poles, but is also present in the mitotic spindle. Moreover, fluorescence measurements suggest that the amount of antigenic γ-tubulin increases during mitosis and that the total amount of γ-tubulin in the spindle is larger than the amount of γ-tubulin in the spindle poles.

**MATERIALS AND METHODS**

**Antibodies**

We raised several polyclonal antibodies against γ-tubulin (Table 1). Among them, all except antibodies from rabbit R38 were able to immunostain γ-tubulin in cell extracts after gel electrophoresis and immunoblotting (Julian et al., 1993). In HeLa cell extracts they labeled only γ-tubulin by immunoblotting. All antibodies, except the antibodies specific to the carboxyl-terminal amino acid region of *Drosophila* γ-tubulin (Zheng et al., 1991), were able to recognize large amounts of overexpressed γ-tubulin on immunoblots. Antibodies were affinity-purified on *Xenopus* γ-tubulin isolated by one-dimensional gel electrophoresis (inclusion bodies from *Escherichia coli* strain RR1 with plasmid pTS235; Stearns et al., 1991), and transferred to a nitrocellulose membrane. After an incubation for 1 hour at room temperature with TBS (10 mM Tris–HCl, pH 7.4, 155 mM NaCl) containing 10% (w/v) milk and 0.1% Tween-20, the membrane was incubated overnight at 4°C with the diluted serum (1/100). After three washes in TBS containing 1% (w/v) milk and 0.1% Tween-20, the antibodies were eluted for 20 minutes in 0.2 M glycine, pH 2.8, 1.0 mM ethylene glycol-bis (β-aminoethylether) N,N,N′,N′-tetraacetic acid (EGTA). Then the medium was neutralized with Tris–HCl, pH 8.0, approximately 0.3 M final (Sambrook et al., 1989). Affinity-purified antibodies were used at a final dilution of 1/5 to 1/10, while immune sera were used at a final dilution of 1/1000 to 1/2000. The rat monoclonal antibody YL1/2 (Kilmartin et al., 1982) was used to reveal α-tubulin while the mouse monoclonal antibody DM1B (Bloise et al., 1984) was used to reveal β-tubulins.

**Immunofluorescent cell labeling**

Four distinct fixation protocols (A-D), schematically described in Table 2, have been applied to cells exponentially grown on glass coverslips. After fixation, cells were reacted for 90 minutes at 37°C with primary antibodies in PBS (3.5 mM PO4KH2/PO4Na2H, 0.13 M NaCl, pH 7.0) with 25% fetal calf serum, washed (2× 10 minutes with 0.05 M Tween-20 in PBS and once in PBS). The staining was revealed with goat anti-rabbit, goat anti-guinea pig, or goat anti-rat antibodies (Nordic) in PBS with 40% fetal calf serum. When double immunolabeling was performed with fluorescein and with rhodamine-labeled secondary antibodies, the amount of secondary antibody labeled with rhodamine was adjusted in such a way that the rhodamine staining was low enough to remain undetectable when observed with the fluorescein set of filters and with the camera settings used for the observation of normal fluorescein labeling. Nuclei and chromosomes were stained with 4′,6-diamidino-2-phenylindole (0.2 µg per ml; DAPI). Preparations were dried, mounted in Mowiol (Rodriguez and Deinhardt, 1960), sealed, and observed by epifluorescence with a Zeiss Axiohot equipped with ×400 (NA, 1.30) and ×1000 (NA, 1.30) Plan-neofluar objectives, an Optovar varying from ×1.25 to ×2.5, a ×4 TV camera and a stabilized excitation beam. Images recorded in the linear dynamic range by a Nocitcon camera (LH 4015 from Lhesa), were digitized (100 frames averaging) with an image processing system (Sapphire from Quantel) and recalculated, without subtracting the fluorescence background, using a linear function (‘Stretch’ program from Quantel). Fluorescence intensities (‘Luminance’ program) and organelle surfaces (‘Threshold’ program) were determined in order to calculate integrated fluorescence values. Mouse oocytes and *Drosophila melanogaster* cells were immunolabeled as previously described (Gueth-Hallonet et al., 1993). In both cases the immunolabeling was observed with a Bio-Rad MRC 600 confocal laser microscope (averaging on 10 images), mounted on an Optiphot II Nikon microscope (×60, NA, 1.40, Plan objectif) equipped with an

<table>
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<th>Code</th>
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<th>Immunizing peptide</th>
<th>γ-Tubulin sequence</th>
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<td>R38</td>
<td>Rabbit</td>
<td>-YEEFATEGDGRKDVS-</td>
<td>38-50 Aspergillus*</td>
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<tr>
<td>R70</td>
<td>Rabbit</td>
<td>-YNIQGEADPTDVKSHL-</td>
<td>323-338 Aspergillus*</td>
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<td>R75</td>
<td>Rabbit</td>
<td>-EYHAATRPDYISWTQEQ</td>
<td>434-451 Human†</td>
</tr>
<tr>
<td>C3</td>
<td>Guinea pig</td>
<td>-EYHAATRPDYISWTQEQ</td>
<td>434-451 Human†</td>
</tr>
<tr>
<td>R62</td>
<td>Rabbit</td>
<td>-KSEDSRVTSAGS</td>
<td>463-475 Drosophila‡</td>
</tr>
</tbody>
</table>

*Antibodies (Julian et al., 1993) raised against the amino acid regions 38-50 and 323-338 of *Aspergillus* γ-tubulin (Oakley and Oakley, 1989) recognized human γ-tubulin (Julian et al., 1993), since the corresponding sequences were -EYEEFATEGDGRKDVS- and -YNIQGEADPTDVKSHL-, respectively (Zheng et al., 1991).

†Antibodies (Julian et al., 1993) raised against the carboxyl amino acid region of human γ-tubulin (Zheng et al., 1991) recognized both mammalian and amphibian γ-tubulins (Zheng et al., 1991; Stearns et al., 1991).

‡Immune serum R62 recognized only γ-tubulin in *Drosophila* cell extracts.
argen ion laser adjusted at 588 nm wave length for fluorescein and a
helium-neon laser adjusted at 543 nm for propidium iodide (2 µg per
ml; PI).

Electron microscopy
PtK2 cells were washed in 80 mM PIPES, pH 6.9, 5 mM EGTA, 1
mM MgCl2 (PEM), permeabilized at 37 °C for 5-15 seconds in PEM
containing 0.2% Triton X-100 and 10 µM taxol, washed (3 minutes
in PEM), fixed (PEM with 0.2% glutaraldehyde, 2% formaldehyde,
for 30 minutes), and incubated with 2% glycine for 30 minutes in
PEM and for 30 minutes in TBS, pH 6.9. Then cells were treated for
immunocytochemistry (immune serum from rabbit R75, 1/300, 37 °C,
3 hours in 25% fetal calf serum; gold-labeled secondary antibody,
37 °C, 90 minutes in 40% fetal calf serum), post-fixed with glu-
taraldehyde (2% in 0.1 M cacodylate buffer, pH 7.2, for 14 hours with
or without 2 mg per ml tannic acid) and processed for electron
microscopy. Serial 100-150 nm thin sections were observed with a
Phillips electron microscope (EM301) at 80 kV.

RESULTS
The presence of γ-tubulin in the mitotic spindle of
HeLa cells was specifically detected by four
different antibodies
Antibodies were affinity-purified, using recombinant Xenopus
γ-tubulin, from the immune serum of a rabbit (R75) immunized
against the 18 carboxy-terminal amino acids of human γ-
tubulin (Table 1; Zheng et al., 1991). These antibodies were
used to stain HeLa cells. They specifically labeled the centro-
some in interphase and the spindle poles in metaphase (Fig. 1)
in agreement with the reported localization of γ-tubulin (Zheng
et al., 1991; Stearns et al., 1991). In addition a weaker staining of
the mitotic spindle was observed. The labeling of the spindle
was also apparent when the fluorescence intensity was
recorded along a straight line going through the two spindle
poles (Fig. 2, metaphase M). The fluorescence was maximal at
each spindle pole, but the peak of fluorescence was clearly
asymmetrical. The fluorescence extended over the entire
spindle, but was absent in the aster. This labeling was absent
when the affinity-purified antibodies were preincubated with
the immunizing peptide (Fig. 1, 75+P), but remained
unchanged when they were preincubated with purified α/β-tubulin heterodimers (Fig. 1, 75T). This fluorescence was
observed in the absence of counterstaining of chromosomes
with DAPI (Fig. 1), thus ruling out the possibility of an arte-
factual staining by this fluorochrome known to interact with
microtubules (Bonne et al., 1985). Identical results were
obtained with the immune serum R75 (1:1000) even after a
preincubation with an E. coli extract (1 mg/ml) devoid of γ-
tubulin or with purified sheep brain α/β-tubulin heterodimers
(0.5 mg/ml, i.e. 5 µM). Conversely, the labeling was not
observed with the preimmune serum and with the immune
serum preincubated either with the immunizing peptide (20
µg/ml, i.e. 9.3 µM) or with an E. coli extract (1 mg protein/ml)
overexpressing recombinant Xenopus γ-tubulin.

Both the immune serum R75 and the corresponding affinity-
purified antibodies reacted only with γ-tubulin in HeLa cell
extracts by immunoblotting. Moreover, these antibodies did
not cross-react with α- and β-tubulins. However, this observa-
tion cannot completely rule out the possibility that the anti-
bodies reacted also with an epitope unrelated to γ-tubulin and
present in the mitotic spindle. In order to prove that the labeling
of the mitotic spindle was due to the presence of γ-tubulin, we
used another immune serum raised against the 18 carboxy-
terminal amino acids of human γ-tubulin in guinea pig (C3)
and two rabbit immune sera (R38 and R70; Julian et al., 1993)
raised against peptides corresponding to two distinct and
conserved γ-tubulin amino acid sequences (Table 1). These
affinity-purified antibodies, which recognized only γ-tubulin
by immunoblotting HeLa cell extracts, led to a labeling
identical to the figures observed with the R75 antibodies.
Metaphase HeLa cells showed a strong staining of the poles
and a weaker labeling of the spindle (Fig. 1). In all cases
staining was inhibited when the antibodies were preincubated
with the corresponding immunizing peptides (Fig. 1, +P), and
remained unchanged when they were preincubated with
purified α/β-tubulin heterodimers (Fig. 1, +T). The relative dis-
tribution of γ-tubulin at the centrosome and in the spindle
was identical with all four affinity-purified antibodies (Fig. 1). Thus
it was clear that γ-tubulin was present in the mitotic spindle of
HeLa cells since the labeling was specifically obtained with
distinct antibodies raised against distinct epitopes of γ-tubulin.

Detection of γ-tubulin in the mitotic spindle is
independent of the fixation procedures
The presence of γ-tubulin in the mitotic spindle could be sig-
ificant or it could result from a relocalization of this protein
during the preparation procedure (Melan and Sluder, 1992). In
order to evaluate the influence of the preparation procedures,
we compared the spindle staining of HeLa cells with affinity-
purified antibodies (R75) after four distinct protocols (Table
2). The specific labeling of the mitotic poles and of the spindle
was observed after fixation with formaldehyde (Fig. 1, 75),
glutaraldehyde (Fig. 1, 75g) and cold methanol (Fig. 1, 75m).
The same figures were also observed when cells were first
extensively permeabilized by Triton X-100 before fixation
with formaldehyde (Fig. 1, 75pf). Some differences in the
overall labeling were noticed between the four different
fixation protocols. After methanol fixation the fluorescence of
the cytoplasm was higher than after fixations with formalde-
hyde and glutaraldehyde (Fig. 1, compare 75m with 75 and
75g). However, in all cases γ-tubulin was always observed not
only at the spindle poles, but also into the spindle.

Quantification of fluorescence intensities raised by anti-
bodies has to be performed cautiously since the fluorescence
could depend on the fixation procedure used and on the depth
of field of the objectives. Taking these difficulties into account,
we compared the fluorescence intensities of γ-tubulin anti-
bodies in the spindle and at the mitotic poles when the images
were taken in the dynamic range of the camera and were not
subjected to non linear image enhancement. For example, in
the metaphase figure shown in Fig. 1 (75), after subtraction of
the overall fluorescence background of the cell, it was clear that
the fluorescence intensity raised by γ-tubulin antibodies
was maximum at the poles. Each spindle pole appeared
strongly labeled, since the average fluorescence intensity
measured at the poles was about 5-6 times higher than the
average fluorescence of the spindle. However, most of the
fluorescence intensity was mainly distributed in the spindle. As
the centrosomal areas represented 4-5% of each half-spindle,
the total fluorescence of the spindle poles accounted for 21-
22% of the overall fluorescence of the mitotic apparatus. Con-
versely, 78-79% of γ-tubulin total fluorescence was distributed in the spindle, although the average fluorescence was 16-19% of the average fluorescence intensity determined at the poles. Thus analyses performed with fluorescent images of γ-tubulin antibodies suggest that about 3/4 of γ-tubulin was present in the spindle, while about 1/4 of γ-tubulin only was present at the poles.

The observation of the low fluorescence intensities in the spindles needed the use of high titer antibodies in order to demonstrate the presence of γ-tubulin. We observed that decreasing twofold the titer of antibodies either in the immune sera or in affinity-purified antibody preparation prevented the observation of the spindle labeling although the staining of the poles was still visible.

The presence of γ-tubulin in the mitotic spindle depends upon the mitotic stage

In order to get an overall view of the evolution of the staining during the cell cycle we compared the fluorescence intensities of HeLa cells during mitosis (Fig. 2). Cells in various mitotic stages were observed in a single immunocytochemical preparation. Fluorescence measurements were made using identical filters, camera setting parameters and linear image treatment parameters. In interphase, γ-tubulin was restricted to the centrosome (Fig. 2, I) and no staining was detected in the microtubule cytoskeleton. Moreover, the intensity of the fluorescence recorded at the centrosomal level was lower than the fluorescence observed at the spindle poles (Fig. 2, left and right columns; compare I and M).

The integrated fluorescence measured on the interphase centrosome [average value of fluorescence intensity] × [centrosome area] corresponded to 7-12% of the total fluorescence recorded at the poles and 0.7% of the total fluorescence recorded in the entire mitotic apparatus (spindle poles and two half-spindles) in metaphase stage (Fig. 2, right column). In prophase, fluorescence intensity was increased at the centrosomal level as the two centrosomes migrated toward opposite sides of the nucleus (Fig. 2, compare I and P). The fluorescence on each centrosome reached about 40-161% of the integrated fluorescence measured at the pole in metaphase. Only a limited fluorescence, shown by the shoulders of the fluorescence peaks, at the centrosomal level, could be detected in the microtubule bundles between the two centrosomes (Fig. 2, P). In prometaphase (Fig. 2, PM), after the disappearance of the nuclear envelope, the integrated fluorescence remained high on the two centrosomes, accounting for 75-128% of the metaphase value (Fig. 2, right column), while the two half-spindles became fluorescent. This fluorescence pattern remained unchanged in metaphase (Fig. 2, M) and early anaphase (Fig. 2, A). In prometaphase and metaphase stages the integrated fluorescence of the spindle accounted for 70-75% and 82-87% of the total fluorescence raised by γ-tubulin antibodies in the mitotic apparatus (Fig. 2, right column). In telophase, the intensity of the fluorescence remained high at the poles, while the two half-spindles and the interzone between the two separated sets of chromosomes exhibited a lower intensity (Fig. 2, T). The integrated fluorescence values of each mitotic pole and of each half-spindle corresponded to 2-3% of the total fluorescence (Fig. 2, right column). In contrast the integrated fluorescence of the interzone corresponded to 90% of the fluorescence. These values undoubtedly have inherent limitations. We noticed that unequal depth of the mitotic apparatuses in the focal plane seemed to constitute an important source of discrepancy in the fluorescence intensity of the two half-spindles. However, the limited quantitative data that are presented are in agreement with qualitative observations of immunofluorescence images. Our results demonstrate that: (1) the amount of γ-tubulin detected by the antibodies on the centrosome increased in prophase and remained at a high level during all stages of mitosis from prophase to telophase (Fig. 2); (2) γ-tubulin was present in the spindle from prometaphase to telophase (Fig. 2, PM-T); and (3) taking into account the respective size of the mitotic poles and of the half-spindles, it appears that the amount of γ-tubulin present in the spindle largely exceeded the amount of γ-tubulin present at the poles (Fig. 2, right column).

Comparative subcellular localization of γ-tubulin and α/β-tubulins

The subcellular localization of γ-tubulin was studied in metaphase stage HeLa cells by double labeling (Fig. 3). Since the amount of γ-tubulin in the cell is far lower than the amount of α/β-tubulin (Sterns et al., 1991), great care was taken to use experimental conditions where no fluorescence overlap could occur between the rhodamine fluorescence raised by the secondary antibodies used to reveal α- or β-tubulin antibodies and the fluorescein fluorescence used to reveal γ-tubulin antibodies. In the conditions used, the fluorescence obtained by the single labeling raised either by a monoclonal rat antibody against α-tubulin or a mouse monoclonal antibody against β-tubulin and rhodamine-labeled secondary antibodies did not give any fluorescence in the window for fluorescein (Fig. 3, compare A and A′), and the fluorescence obtained by a single labeling with the rabbit affinity-purified antibodies against γ-tubulin (R75) and fluorescein-labeled secondary antibodies did not give any fluorescence in the window of rhodamine (Fig. 3, compare B and B′). Moreover, when the double-labeling was performed in the presence of the antigenic peptide the α- and β-tubulin stainings were not modified while the staining of γ-tubulin could no longer be detected (Fig. 3E and E′).

It is not possible by double staining to compare the absolute intensities of fluorescence raised by two distinct antibodies directed against different proteins, but it is possible to compare their relative intensities (Fig. 3D). It is clear that the staining of γ-tubulin was maximal at the centrosome level. It decreased in the mitotic spindle to reach the background level at the chromosomal metaphase plate (Fig. 3C′ and arrow in D). In contrast the fluorescence raised by α- or β-tubulins was maximal in the spindle itself and not at the centrosomal level. Thus the fluorescence patterns of γ- and α/β-tubulins were not coincidental. Although γ-tubulin was present in the mitotic spindle, we failed to detect γ-tubulin in the microtubules that composed the aster at the spindle poles. Even when the γ-tubulin fluorescence pattern was recalculated using a non linear function (Histogram, Quantel) in order to visualize even a faint staining of these microtubules, no γ-tubulin could be detected at the level of the extra spindle microtubules of the aster (not shown).

Using the same conditions of double labeling, we studied whether the integrity of microtubules was necessary for the presence of γ-tubulin in the mitotic spindle. HeLa cells were permeabilized in the presence of calcium ions before fixation.
Fig. 1. γ-Tubulin staining in HeLa cells with four different affinity-purified antibodies and four fixation procedures. Four antibodies affinity-purified from rabbit 75, guinea pig C3, rabbit 70 and rabbit 38 have been used (formaldehyde fixation: procedure A in Table 2) in the absence of DAPI counterstaining without preincubation (75, C3, 70, 38) and after preincubation with the corresponding antigenic peptide (P; 20 µg per ml, i.e. 9.3 µM) or with purified sheep brain tubulin (T; 0.5 mg/ml, i.e. 5 µM). Alternatively, cells were prepared according to three other different procedures (B, C and D in Table 2) and labeled with affinity-purified antibodies (R75) without chromosome counterstaining with DAPI. Metaphase stages corresponding to the different preparation procedures are shown in images 75pf (procedure B: permeabilization and formaldehyde fixation), 75g (procedure C: fixation with glutaraldehyde), and 75m (procedure D: cold methanol fixation).
Then the subcellular distributions of α- or β-tubulins and γ-tubulin were compared. In interphase cells, all microtubules were disassembled and γ-tubulin was exclusively located at the centrosome. In early mitotic cells (late prophase, prometaphase and metaphase stages) submitted to a permeabilization in the presence of calcium ions, most mitotic microtubules were disassembled. α-, β- and γ-tubulin antibodies labeled one or two aster-like figures. Comparison of the staining demonstrated a complete coincidence between the figures raised by α- or β-tubulin antibodies and γ-tubulin antibodies (Fig. 4, compare A and A’, D and D’). In late mitotic cells (late anaphase and telophase stages), the overall picture was slightly different. In these cells a coincidental staining of α- or β-tubulin antibodies and γ-tubulin antibodies was observed in the two remnants of the spindle (Fig. 4). However, calcium-stable microtubules stained by α- and β-tubulin antibodies in the interzone between the two sets of chromosomes were apparently entirely devoid of γ-tubulin (Fig. 4, compare B and B’). Electron cytochemistry confirmed that γ-tubulin was associated with spindle microtubules insensitive to the action of calcium, which mainly corresponded to kinetochore microtubules (Fig. 5). Thus, after microtubule disassembly in the presence of calcium, γ-tubulin was clearly associated with the α/β-tubulin heterodimers of the residual spindle, while it was absent in interzonal microtubules.

**γ-Tubulin is present along mitotic microtubules**

Visualizing ultrastructural localization of γ-tubulin in the spindle could be of interest for understanding its physiological role. Specific gold particle labeling of serum R75 antibodies confirmed the presence of γ-tubulin in the spindle (Fig. 5, upper panel, 75). The gold particles were no longer observed when the serum was preincubated with the immunizing peptide (Fig. 5, upper panel, 75+P). In order to determine whether γ-tubulin was localized at the microtubule extremities or along the microtubules, we studied the relative localization of gold particles and microtubules in serial 100-150 nm thin sections. It was not possible to determine whether all gold particles were localized along microtubules and we cannot exclude the possibility that gold particles could be localized in the spindle without any interaction with microtubules. Similarly, due to oblique sectioning of microtubules, we could not exclude the possibility that some gold particles were localized at the microtubule ends. However, it is clear that gold particles were seen along microtubules, since in numerous cases no microtubule ends could be detected in the previous and in the following sections (Fig. 5, lower panel). Moreover, the distance of the gold particles to the microtubules was consistent with the presence of γ-tubulin at the surface of the microtubule walls.

**The presence of γ-tubulin in the mitotic spindle is observed in evolutionarily distant animal cells**

Using both immune sera (C3 and R75) and affinity-purified antibodies (C3 and R75) against γ-tubulin, we specifically labeled not only the spindle poles, but also the metaphase spindle in PtK2 cells (Fig. 6, PtK2). The staining was abolished when the immune sera were preincubated with the immunizing peptide and *E. coli* extracts containing overexpressed *Xenopus* γ-tubulin, but remained unchanged when the immune sera were preincubated with purified sheep brain tubulin and *E. coli* extract devoid of γ-tubulin (not shown). Similarly the staining was abolished when affinity-purified antibodies were preincubated with the immunogenic peptide (Fig. 6, PtK2, 75+P) but remained unchanged when they were preincubated with sheep brain tubulin (Fig. 6, PtK2, 75+T). Identical results were obtained with all four fixation procedures (Table 2) with PtK2 cells (not shown). These affinity-purified antibodies stained the metaphase spindles in J7 mouse cells, Chinese

### Table 2. Different fixation protocols used

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<th>Fixation</th>
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<td><strong>A</strong></td>
<td><strong>B</strong></td>
<td><strong>C</strong></td>
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<tr>
<td>–</td>
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<td>–</td>
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<tr>
<td>–</td>
<td>3.0% Formaldehyde pH 6.9 and pH 10.0</td>
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<tr>
<td>–</td>
<td>0.25% Glutaraldehyde pH 6.9 and sodium borohydride</td>
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<td>–</td>
<td>Cold methanol and rehydration</td>
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*a* Protocol A: fixation for 15 minutes at room temperature in 3.7% formaldehyde in PBS, pH 7.2, and then for 45 minutes in 3.7% formaldehyde in 50 mM carbonate buffer, pH 10; three washes for 5 minutes in PBS; permeabilization for 6 minutes in cold acetone (−20°C); three washes in PBS.

†Protocol B: five washes in 100 mM PIPES, pH 6.9, 1 mM EGTA, 2 mM MgCl2, 4% polyethylene glycol 6000 (PEM-PEG, pH 6.9); permeabilization for 1 minute at 22°C in PEM containing 0.5% Triton X-100; five washes for 5 minutes in PEM-PEG; fixation for 15 minutes in 3.0% formaldehyde in PEM containing 1% dimethyl sulfoxide and then for 205 minutes in 3.0% formaldehyde in 50 mM carbonate buffer, pH 10; final wash in PBS.

‡Protocol C: five washes for 5 minutes in PEM, pH 6.9; fixation for 10 minutes in 0.25% glutaraldehyde in PEM; three washes for 5 minutes in PBS, pH 7.2. Three incubations for 10 minutes in PBS, pH 8.0, containing 10 mg per ml sodium borohydride; three washes for 5 minutes in PBS, pH 7.2; permeabilization for 6 minutes in cold acetone (−20°C); three washes for 5 minutes in PBS.

§Protocol D: fixation for 5 minutes in cold (−20°C) methanol containing 1 mM EGTA; rehydration at room temperature in PBS containing 75%, 50% and 25% methanol; three washes for 5 minutes in PBS.

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**Fig. 2.** Variation of γ-tubulin staining in HeLa cells as a function of the cell stage. I, interphase; P, prophase; PM, prometaphase; M, metaphase; A, early anaphase; T, telophase. Left column, staining raised by affinity-purified γ-tubulin antibodies (C3); all images were obtained on the same preparation using identical conditions. Middle column, mitotic stages determined by DAPI counterstaining. Right column, luminance (recorded in arbitrary linear units) has been determined (Luminance program from Quantel) along a straight line passing through the two poles of the mitotic apparatus. Alternatively, the average fluorescence and the surface have been determined over the centrosomal and spindle areas defined using the threshold function (Quantel) in order to calculate the integrated fluorescence intensities (laverage fluorescence intensity×[surface]) of the centrosomal and spindle areas, respectively (the two recorded values correspond to the two spindle poles and the two half-spindles).
hamster ovary cells, and Xenopus XCT cells (Fig. 6, J7, CHO and XCT). In all cases the labeling was specific, since it was prevented by preincubation of the antibodies with the immunizing peptide (Fig. 6, +P), but remained unchanged when preincubated with α/β-tubulin (Fig. 6, +T). Similarly, using the immune sera R62, that we have raised against the 13 carboxy-terminal amino acids of Drosophila melanogaster γ-tubulin (Table 1; Zheng et al., 1991), we observed on the Drosophila cells (Kc diploid cell line; Echalier and Ohanessian, 1970) a specific staining of the poles and of the mitotic spindle (Fig. 6). Thus, the presence of γ-tubulin in the metaphase spindle is general in animal cells, since it is observed in distant vertebrates from human to Xenopus and in insect cells. However, using the same antibodies the fluorescence varied according to the cell type. For example, the intensity observed in J7 mouse cells with the serum R75 (Fig. 6, J7) was lower than that observed in mouse oocytes (see Fig. 2 in Gueth-Hallonet et al., 1993). Moreover, although γ-tubulin was found in the spindle from prometaphase to early telophase in HeLa, PtK2, and Drosophila cells, it could be detected only in metaphase in mouse, Chinese hamster ovary and Xenopus XCT cells (data not shown). This inability to detect a specific labeling in

![Fig. 3. Comparison of the labeling of HeLa cells by γ- and α-tubulin antibodies. The rat monoclonal antibodies against α-tubulin (YL) were revealed by rhodamine-labeled secondary antibodies: the staining appeared in the window used to observe rhodamine staining (YL; A'), while no fluorescence was recorded in the window used for the observation of fluorescein staining (A). Conversely, the rabbit affinity-purified antibodies R75 were revealed by fluorescein-labeled secondary antibodies: the staining appeared in the window used for fluorescein (75; B), while no fluorescence was recorded in the window used to observe the fluorescence of rhodamine (B'). Using double labeling, the images corresponding to γ- and α-tubulins are shown in C (75; fluorescein) and C' (YL; rhodamine), while chromosomes staining is shown in C'' (DAPI). The variations of intensities (I in arbitrary units) raised by γ-tubulin antibodies (γ in C), α-tubulin antibodies (α in C'), and DAPI (C'', arrow in D), have been quantified (luminance program from Quantel) along a straight line passing through the two poles of the mitotic apparatus (D). Comparison of C and C' shows that the spindle is labeled by both γ- and α-tubulin antibodies, while the other microtubules of the aster (arrows in C) are labeled by the α-tubulin antibodies only. The fluorescence raised by γ-tubulin antibodies is prevented when antibodies were preincubated with the antigenic peptide (E; 75+P), while the fluorescence raised by the α-tubulin antibodies remained unaffected (E').]
prometaphase and anaphase in these cells did not result from the inability of the antibodies to reveal low levels of \(\gamma\)-tubulin as shown by the clear labeling of the interphase centrosomes in these cells. These results suggest that \(\gamma\)-tubulin is present in the metaphase spindle of animal cells but that the extent of the presence of \(\gamma\)-tubulin in the spindle depends on the cell type.

**DISCUSSION**

\(\gamma\)-Tubulin has been described in the spindle of plant cells (Liu et al., 1993). Its localization in the spindle of animal cells has not yet been reported (Zheng et al., 1991; Stearns et al., 1991; Joshi, 1993). Here, we have specifically labeled \(\gamma\)-tubulin in...
the spindle of a variety of animal cells (Figs 1 and 6) using several different affinity-purified antibodies (Table 1) and preparation procedures (Table 2; Fig. 1). Identical staining of the mitotic spindle was observed with all antibodies, thereby ruling out the possibility of a cross reaction with a protein unrelated to γ-tubulin. In all cases, preincubation of the antibodies with the immunizing peptides prevented the labeling, but preincubation with purified α/β-tubulin heterodimers did not change the staining. Hence, the γ-tubulin antibodies do not cross react with the α/β-tubulin heterodimers, in agreement with the absence of sequence homology between the immunizing peptides and the α- and β-tubulins. We failed to detect γ-tubulin in the astral microtubules, which do not participate in the structure of the spindle. Thus, artefactual relocalization of γ-tubulin from the poles to the mitotic spindle during the preparation procedure is unlikely (Melan and Sluder, 1992).

Fig. 5. Ultrastructural localization of γ-tubulin. Upper and middle panels (75 and 75+p): HeLa cells have been permeabilized in the presence of Ca²⁺, and processed for immunoelectron microscopy. 75: treatment with antibodies R75; gold particles are visible on kinetochore microtubules (mt). 75+p: treatment with antibodies R75 preincubated for 2 hours at 37°C with the immunizing peptide (200 µg/ml, i.e. 93 µM); no gold particles are observed. ch, chromatin; k, kinetochore; ce, centriole. Lower panel (75A,B,C), ultrastructural localization of γ-tubulin revealed by the antibodies R75 in the mitotic spindle of PtK2 cells. A gold particle is observed in the near proximity of a microtubule wall (thin section B, broken lines, microtubule axis). This localization cannot be explained by the presence of a microtubule extremity since the sections of the same microtubule were observed in the two adjacent thin sections A and C. No labeling was observed when the immune serum (R75) was preincubated with the antigenic peptide (not shown).
Fig. 6. Variation of γ-tubulin staining in cells from several different animals. CHO, Chinese hamster ovary cells (antibodies R75); J7, mouse cells (antibodies R75); PtK2, kangaroo rat cells (antibodies R75); XCT, Xenopus cells (antibodies C3), and Kc, Drosophila cells (immune serum R62 specific for the carboxy-terminal amino acid sequence of Drosophila melanogaster γ-tubulin). Alternatively, affinity-purified antibodies (R75 and C3) or the immune serum R62 were preincubated with the antigenic peptide (P) and with purified sheep brain tubulin (T). Chromosomes were not counterstained except in the case of CHO (DAPI), XCT cells (DAPI), and Drosophila cells (propidium iodide, PI).
We first thought that different preparation procedures might account for the failure of previous immunological studies to detect γ-tubulin in the mitotic spindle. However, no major labeling differences were observed with a variety of fixation and permeabilization schemes (Table 2). On the other hand it is clear that the fluorescence intensity per unit area obtained with γ-tubulin antibodies is higher at the poles than in the mitotic spindle. Hence, a lower titer of the antibodies may have prevented the clear observation of γ-tubulin in the spindle in previous studies.

We made identical observations in a variety of cell types such as human cells (Fig. 1), PtK2 cells, mouse cells, mouse oocytes, Chinese hamster ovary cells, Xenopus and Drosophila cells (Fig. 6). The intensity of the staining depended on the species. It was more pronounced in HeLa, PtK2, and Drosophila cells than in mouse, Chinese hamster ovary cells and Xenopus cells. Similarly, for a given species the labeling of the spindle by a particular antibody varied according to the cell type. For example, the staining of the mitotic spindle was lower in mouse cells grown in culture than in mouse oocytes.

The quantity of antigenic γ-tubulin in the mitotic spindle varied with the mitotic stage. In all cell types, we failed to detect γ-tubulin in the interphasic microtubule cytoskeleton except at the centrosome. In HeLa and PtK2 cells, no significant fluorescence intensity was recorded in early microtubule asters assembled in prophase although the labeling of the centrosome was intense. Spindle localization of γ-tubulin became apparent in prometaphase, reached a maximum in metaphase and disappeared in late telophase (Fig. 2). In contrast, in mouse, Chinese hamster and Xenopus cells (Fig. 6), the staining of the spindle by γ-tubulin antibodies was restricted to the metaphase stage.

Previously reported exclusive localization of γ-tubulin at the centrosome and at the minus extremities of microtubules (Zheng et al., 1991; Stearns et al., 1991; Joshi, 1993) suggested that γ-tubulin might constitute a specific probe of the centrosome surface. In this model the staining of the mitotic spindle could correspond to numerous thin centrosomal appendages extending into the spindle as a non-microtubule matrix component (Mitchison, 1992). Alternatively microtubules nucleated on the centrosome could detach from the nucleating organelle with γ-tubulin at their minus ends (Mcbeath and Fujiiwa, 1990). However, the exclusive localization of γ-tubulin at the minus extremities of spindle microtubules was not confirmed by electron cytochemistry in serial thin sections (Fig. 5). Electron microscopic localization of the spindle γ-tubulin along the microtubules suggests that at least part of the γ-tubulin could also interact with the microtubule walls, in contrast to previous in vitro observations (Melki et al., 1993; Marchesi and Ngo, 1993). Furthermore, after calcium-induced disassembly of microtubules, our electron microscopy measurements revealed that the aster-like figures that could be stained with γ-, α- and β-tubulin antibodies corresponded to microtubules. These results indicate that γ-tubulin is a constitutive part of the mitotic spindle. Localization of γ-tubulin on the spindle might reflect diffusion along spindle microtubules of either γ-tubulin or multimolecular protein complexes containing γ-tubulin (Raff et al., 1993; Marchesi and Ngo, 1993). Alternatively γ-tubulin could play a role in microtubule dynamics and could act in the stabilization of some microtubules such as kinetochore microtubules. This latter view is consistent with the initial characterization of γ-tubulin by a screening procedure intended to detect proteins capable of interacting with microtubules and modifying their stability (Weil et al., 1986; Oakley and Oakley, 1989). We observed that γ-tubulin remains associated with kinetochore microtubules after cell permeabilization in the presence of calcium ions suggesting that γ-tubulin might act as a microtubule stabilizing protein.

The observed alteration in the localization and concentration of antigenic γ-tubulin may represent increased concentrations of γ-tubulin at the mitotic structure or the exposure of antigenic protein sites as a result of functional conformational changes. Although no results support a differential cell cycle-dependent antigenicity, we observed that γ-tubulin staining appeared after a prolonged fixation in formaldehyde, suggesting a modification of γ-tubulin epitopes accessibility to antibodies. On the other hand, identical results were observed with antibodies directed at three widely separated sequences of the protein (Table 1). It seems unlikely that precise functional changes would alter the antigenicity of many protein domains. The amount of antigenic γ-tubulin detected in the interphase centrosome is far below the amount observed in the entire mitotic apparatus (1-0.7%) or even at a spindle pole (7-12%). However, immunoblotting of total cell extracts, made in mitosis and 2, 4, 6, 8 hours after mitosis of PtK2 cells synchronized by selective detachment, failed to detect a large variation of the total pool of γ-tubulin during the cell cycle (unpublished data). These observations could suggest that the immunofluorescence protocols used so far have allowed the observation of a subpopulation of γ-tubulin that participates directly in the structure and/or in the dynamics of the microtubule cytoskeleton, and that the soluble pool of γ-tubulin is lost during the permeabilization procedures. In agreement with this hypothesis the fluorescence intensity of the cytoplasm was higher after methanol fixation (Fig. 1, 75m) than after fixation with formaldehyde and glutaraldehyde (Fig. 1, 75 and 75g).

Taken together, these results argue for a recruitment of γ-tubulin to the centrosome and the spindle apparatus during mitosis.

In summary, the distribution of γ-tubulin in animal cells is more complex than initially assumed (Oakley et al., 1990; Oakley, 1992). In interphase cells γ-tubulin is present in the centrosome but remains undetected in the microtubule cytoskeleton (Zheng et al., 1991; Stearns et al., 1991). During mitosis the amount of γ-tubulin observed in the spindle poles is much larger than the amount detected in the interphase centrosome. Antigenic γ-tubulin appears at the spindle during mitosis where it is localized at the surface of microtubules. In contrast, γ-tubulin remains undetected in the short aster microtubules. Finally, during cytokinesis, we have shown previously that γ-tubulin is not only present in the centrosome, but is localized in the minus regions of the interdigitated microtubules constituting the midbody (Julian et al., 1993). The cell stage distribution of γ-tubulin varies between animal cells of different species and between cells of different tissues within the same species. These observations imply that both the localization and concentration of γ-tubulin are highly regulated during the cell cycle and that the regulation may vary between cell types and between species.

Our understanding of the mechanism of action of γ-tubulin is at a very early stage and much remains to be learned. We
have found recently that cellular γ-tubulin is not only present in a readily centrifugable form but also in a soluble form that could be present, at least partially, in multimolecular complexes (Weil et al., 1986; Oakley and Oakley, 1989). In addition, several γ-tubulin isotypes have been observed in mammalian cell extracts by two-dimensional gel electrophoresis (L. Lajoie-Mazenc and M. Wright, unpublished results). While it is not yet possible to correlate the cell stage-dependent subcellular localizations of γ-tubulin observed in this report with the preliminary evidence showing the complex biochemical features of this protein, it is clear that the physiological role of γ-tubulin is likely more diversified than previously assumed.

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