Association of brain γ-tubulins with $\alpha\beta$-tubulin dimers

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γ-Tubulin is necessary for nucleation and polar orientation of microtubules *in vivo*. The molecular mechanism of microtubule nucleation by γ-tubulin and the regulation of this process are not fully understood. Here we show that there are two γ-tubulin forms in the brain that are present in complexes of various sizes. Large complexes tend to dissociate in the presence of a high salt concentration. Both γ-tubulins co-polymerized with tubulin dimers, and multiple γ-tubulin bands were identified in microtubule protein preparations under conditions of non-denaturing electrophoresis. Immunoprecipitation experiments with monoclonal antibodies against γ-tubulin and α-tubulin revealed interactions of both γ-tubulin forms with tubulin dimers, irrespective of the size of complexes. We suggest that, besides small and large γ-tubulin complexes, other molecular γ-tubulin form(s) exist in brain extracts. Two-dimensional electrophoresis revealed multiple charge variants of γ-tubulin in both brain extracts and microtubule protein preparations. Post-translational modification(s) of γ-tubulins might therefore have an important role in the regulation of microtubule nucleation in neuronal cells.

Key words: antibody, charge variant, microtubule protein, tubulin complex.

INTRODUCTION

Microtubules, assembled from α- and β-tubulin heterodimers, are highly dynamic polymers that are indispensable for many cellular functions, such as intracellular organization, ordered vesicle transport and cell division, to name but a few. To accomplish their specialized functions, microtubules are organized into complex arrays by a microtubule-organizing centre (MTOC), which, in animal cells, consists of a pair of centrioles surrounded by electron-dense pericentriolar material. Microtubules are nucleated at the pericentriolar material by their 'minus ends' [1]. One of the key components of MTOCs is γ-tubulin [2].

Although γ-tubulin participates in nucleation of microtubules from MTOCs [3,4], the majority of it is associated with other centrosomal proteins in soluble cytoplasmic complexes. Large γ-tubulin-ring complexes (γTuRCs) were first identified in *Xenopus* eggs [5]. They were also found in *Drosophila* embryos [6] and in various types of mammalian cells [7–9]. Besides γTuRCs, there also exist smaller complexes [γ-tubulin small complexes (γTuSCs)] [10], which comprise two molecules of γ-tubulin and one molecule each of GCP2 and GCP3 (γ-tubulin-complex proteins) [11,12] and which are homologues of the *Saccharomyces cerevisiae* proteins Spc97p and SPc98p associated with spindle pole bodies [13]. The γTuRCs are formed by small complexes and by other proteins. In addition to nucleation from the MTOC, the γTuRCs are also involved in regulating the dynamics of the microtubule minus ends [14].

The γTuRCs have different sizes, and they also differ in protein composition. The question is still open as to whether $\alpha\beta$-tubulin dimer is an integral component of the large complexes. Although, in the majority of analysed complexes, tubulin dimers were not found [15], they were detectable in frog oocytes [5,16], mammalian brains [9] and in a lymphoblastic cell line [8].

γ-Tubulin itself has been found to be a microtubule minus-end binding molecule in not-anchored microtubules [17], and binding sites for α- and β-tubulin subunits on γ-tubulin were identified using synthetic peptides [18]. Structural models for self-assembly and microtubule interactions suggest that γ-tubulin should be capable of self-assembling into dimer or protofilament-like oligomers, as well as of interacting laterally with α- or β-tubulin [19]. Experiments *in vitro* showed that, under some circumstances, γ-tubulin might bind to surface regions of microtubules without affecting the tubulin polymerization [18]. An association of γ-tubulin along microtubule arrays *in vivo* is well described in plants [20,21].

While a number of studies have concentrated on the characterization of proteins that associate with γ-tubulins, and on positioning of γ-tubulin at the minus end of microtubules, our knowledge about the regulation of nucleation activities of γ-tubulin by post-translational modifications is very limited [22].

Because brain tubulin is used typically to study the microtubule dynamics, and the heterogeneity of $\alpha\beta$-tubulin dimers is known in detail, we have looked for the presence of γ-tubulin forms in pig brain. Here, we report for the first time on two γ-tubulin isoforms, which persist at differently sized complexes and interact with $\alpha\beta$-tubulin heterodimers, irrespective of the size of complexes. Brain γ-tubulin is post-translationally modified, and these modifications could have an important role in the regulation of microtubule nucleation.

EXPERIMENTAL

Materials

Immobilized Protein A Plus, Immobilized Protein L Plus and SuperSignal WestPico Chemiluminescent reagents were bought from Pierce (Rockford, IL, U.S.A.). Protease-inhibitor cocktail tablets ('Complete EDTA-free') were from Roche Molecular Biochemicals (Prague, Czech Republic), molecular-mass markers for SDS/PAGE were obtained from Sigma–Aldrich (Prague, Czech Republic), markers for two-dimensional electrophoresis and Biolyte 3/10 were from Bio-Rad Laboratories (Munich, Germany), CNBr-activated Sepharose 4B, IPG buffer (pH 4–7),

Abbreviations used: MTP-2 and MTP-3, microtubule protein after two or three cycles respectively of assembly and disassembly; MTOC, microtubule-organizing centre; γTuRC, γ-tubulin-ring complex; γTuSC, γ-tubulin small complex.

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molecular-mass markers for native electrophoresis and carbamylyte calibration kit for isoelectric focusing were purchased from Amersham Biosciences (Uppsala, Sweden). Peptides were prepared in the Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Prague, as described previously [23].

Antibodies

The following monoclonal antibodies were used: TU-01 (IgG1) [24] and TU-16 (IgM) against α-tubulin [25]; TU-06 (IgM) against β-tubulin [26]; TU-31 (IgG2b) and TU-32 (IgG1) anti-peptide antibodies prepared against the EYHAATRPDYISW-GTQ peptide corresponding to human γ-tubulin sequence 434–449 [23]; GTU-88 (IgG1) anti-peptide antibody prepared against the EEFATEGTDRKDVFFY peptide corresponding to human γ-tubulin sequence 38–53 (Sigma cat. no. T6557); VI-01 (IgM) against vimentin [27]; and N27F3-4 (IgG1) against heat-shock protein Hsp70 (StressGen; cat. no. SPA-820). Actin was detected by affinity-purified rabbit anti-actin antibody (Sigma cat. no. A2066). Anti-mouse immunoglobulin and anti-rabbit immunoglobulin antibodies conjugated with horseradish peroxidase, as well as anti-mouse immunoglobulin conjugated with alkaline phosphatase, were purchased from Promega Biotec (Madison, WI, U.S.A.).

Protein preparation

Microtubule protein (MTP) was purified from pig brain by two temperature-dependent cycles of assembly and disassembly [28]. A third polymerization was performed in the presence of DMSO to partially deplete the microtubule-associated proteins [29], before subsequent purification of tubulin by phosphocellulose chromatography [30]. The high-speed extract for gradient centrifugation was prepared by homogenization of washed brains in cold PEM buffer [100 mM Pipes (pH 6.9)/1 mM EGTA/2 mM $MgSO_4$/1 mM 2-mercaptoethanol] supplemented with 1 mM ATP, 1 mM 4-(2-aminoethyl)benzenesulphonyl fluoride and protease inhibitor cocktail. The homogenate was centrifuged at 41000 ***g*** for 20 min at 4 °C. The supernatant was then re-centrifuged at 280000 ***g*** for 30 min at 4 °C, and immediately subjected to sucrose-gradient centrifugation. When the presence of salt was required, the high-speed extract was supplemented with 500 mM NaCl. Carboxyamidomethylation of MTP-2 was performed as described previously [31]. Protein concentration was determined by means of the bicinchoninic acid kit (Sigma–Aldrich) with BSA as the standard.

Sucrose-gradient centrifugation

Sucrose-gradient centrifugation was performed as described previously [10]. The sucrose gradients (5–40 % in PEM buffer) were poured as step-density gradients (800 μl steps: 40, 30, 20, 10 and 5 % sucrose), and allowed to diffuse into continuous gradients overnight at 4 °C before use. In the case of salt-containing extracts, the sucrose gradient was supplemented with NaCl to a final concentration of 500 mM. A 75 μl aliquot of high-speed extract was loaded on to each gradient, and the gradients were centrifuged at 50000 rev./min in a Beckman SW60 rotor for 4 h at 4 °C. Gradients were fractionated from the top into 14 fractions. Protein standards (BSA, bovine liver catalase and mouse IgM) at the same concentration (0.5 mg/ml each) were loaded in an equivalent volume, and were run in parallel over identical gradients. Samples for SDS/PAGE were prepared by mixing with 2 × concentrated SDS/PAGE sample buffer.

Immunoprecipitation

Immunoprecipitation was performed as described previously [32], using TBST [10 mM Tris/HCl (pH 7.4)/150 mM NaCl/0.05 % (v/v) Tween 20] for dilution of extracts and for washings. High-speed pig brain extracts were incubated with beads of: (i) immobilized Protein A saturated with anti-(γ-tubulin) antibody TU-31; (ii) immobilized Protein A saturated with control antibody NF-09; (iii) immobilized Protein A alone; (iv) immobilized Protein L saturated with anti-(α-tubulin) antibody TU-16; (v) immobilized Protein L saturated with control anti-vimentin antibody VI-01; or (vi) immobilized Protein L alone. The antibodies were used in the form of culture supernatants to avoid immobilization of other mouse antibodies. Sedimented beads (50 μl) were incubated with shaking at 4 °C for 2 h with 1.2 ml of the corresponding antibody, prepared by mixing 0.4 ml of 10 × concentrated supernatant with 0.8 ml of the TBST buffer. The beads were pelleted by centrifugation at 5000 ***g*** for 1 min, washed four times (5 min each wash) in cold TBST, and incubated further with shaking for 3 h at 4 °C with 1 ml of pig brain high-speed supernatant diluted 1:7 with TBST. The beads were then pelleted and washed four times (5 min each) in cold TBST, before boiling for 5 min in 100 μl of SDS sample buffer to release the bound proteins. Alternatively, the TU-31 antibody, purified from ascitic fluid on Protein A, was directly bound to CNBr-activated Sepharose 4B and used for precipitation.

Gel electrophoresis and immunoblotting

SDS/PAGE, electrophoretic transfer of separated proteins on to nitrocellulose and details of the immunostaining procedure are described elsewhere [33]. Under routine conditions, lower-grade SDS (Sigma cat. no. L-5750; 66 % SDS) was used throughout the whole procedure, because it facilitates the separation of α- and β-tubulin subunits [34]. Alternatively, high-grade SDS (Sigma cat. no. L-6026; 99 % SDS) was used, as indicated in the text. The antibodies TU-01, TU-06 and TU-32 in the form of spent culture supernatants were used undiluted, whereas the antibody GTU-88, in the form of ascitic fluid, was diluted 1:4000. The antibody against Hsp70 was diluted 1:2000, and the antibody against actin was diluted 1:200. After incubating the samples with secondary antibodies conjugated with horseradish peroxidase diluted 1:10000, followed by washing, bound antibodies were detected by a chemiluminescence reaction, according to the manufacturer's instructions. Autoradiography films X-Omat AR were from Eastman Kodak (Rochester, NY, U.S.A.). Alternatively, bound antibodies were detected with secondary antibodies conjugated with alkaline phosphatase diluted 1:7500 and chromogenic substrates [33].

In some experiments, the TU-32 antibody was pre-absorbed with the peptide used for immunization, i.e. the 16-amino-acid peptide EYHAATRPDYISWGTQ that corresponds to amino acid residues 434–449 of human γ-tubulin. The other 16-amino-acid peptide, EEFATEGTDRKDVFFY, corresponding to amino acid residues 38–53 of human γ-tubulin, was used as a negative control. Two molar ratios of antibody to peptide were used: 1:5 and 1:50. Mixtures of antibody and peptides were incubated for 30 min at room temperature.

Non-denaturing PAGE was performed using the Laemmli system [35], except that SDS was completely omitted and electrophoresis was performed at 4 °C. Sample buffer consisted of 62.5 mM Tris/HCl, pH 6.8, 10 % (v/v) glycerol and 0.01 % (w/v) Bromophenol Blue.

Two-dimensional PAGE was performed essentially as described previously [36]. Samples (30–50 μg of protein) were diluted with IPG sample buffer containing 7 M urea, 2 M

thiourea, 20 mM Tris, 4% (w/v) CHAPS, 1% (w/v) Triton X-100, 1% (w/v) dithiothreitol and 0.8% (w/v) Biolyte 3/10. Immobiline DryStrip gels with a linear pH 4–7 gradient, 11 cm long (Amersham Biosciences), were rehydrated using 250 μl of prepared sample (in IPG sample buffer). Each strip was overlaid with mineral oil, and left overnight at room temperature. Strips were focused for a total of 22.2 kV · h on Multiphor II apparatus (LKB, Bromma, Sweden). The second dimension was performed using SDS/8.5% (w/v) polyacrylamide gels, and separated proteins were transferred on to nitrocellulose by electroblotting.

RESULTS

Distribution of γ-tubulin in brain extracts and MTPs

The distribution of γ-tubulin was monitored during isolation of pig brain MTP by repeated temperature-dependent cycles of polymerization and depolymerization. The third polymerization occurred in the presence of DMSO, which partially depleted the microtubule-associated proteins (Figure 1A, lane 4), before the purification of αβ-tubulin dimers on phosphocellulose (Figure 1A, lane 5). γ-Tubulin was present in supernatants after low-speed (Figure 1B, lane 1) and high-speed (Figure 1B, lane 2) centrifugation of pig brain extract, as well as in MTP prepared by two (Figure 1B, lane 3) and three cycles (Figure 1B, lane 4) of temperature-dependent polymerization/depolymerization (MTP-2 and MTP-3 respectively). Under the conditions employed, which partially stripped away the high-molecular-mass microtubule-associated proteins, γ-tubulin was still detectable (Figure 1B, lane 4). During the isolation, γ-tubulin was not enriched in MTPs, and no γ-tubulin was detected in purified αβ-tubulin dimers separated from microtubule-associated proteins by chromatography on phosphocellulose (Figure 1B, lane 5). It was possible to elute γ-tubulin, like other microtubule-associated proteins, from the column with 1 M KCl. The same staining pattern was obtained with antibodies against the conserved human γ-tubulin amino acid regions 38–53 (GTU-88) and 434–449 (TU-31 and TU-32).

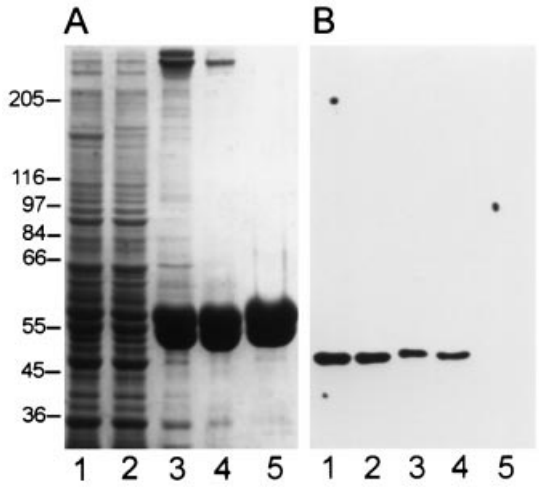

Figure 1 Distribution of γ-tubulin during isolation of MTPs

(**A**) Coomassie Blue staining of separated proteins. Low-speed supernatant of brain extract (lane 1), high-speed supernatant of brain extract (lane 2), MTP-2 (lane 3), MTP-3 (lane 4) and tubulin (lane 5) were separated on SDS/7.5% polyacrylamide gels. (**B**) Immunostaining with anti-(γ-tubulin) antibody TU-32. Aliquots of protein (10 μg) were loaded in lanes 1–4, and 12.5 μg of protein in lane 5. Molecular-mass markers (in kDa) are indicated to the left of (**A**).

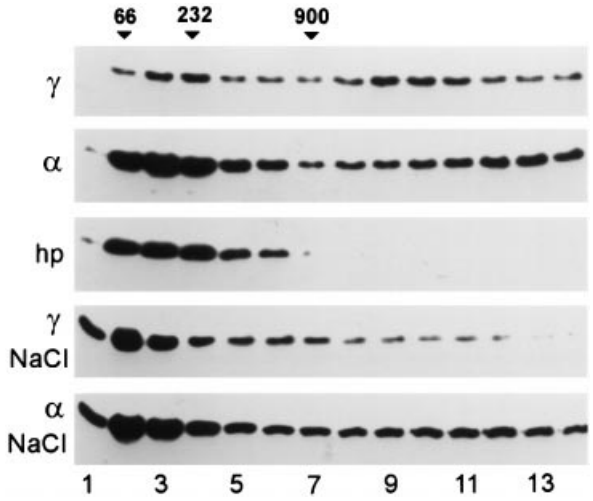

Figure 2 Behaviour of γ-tubulin during sucrose-density gradient centrifugation

Samples were sedimented through a 5–40% sucrose gradient without or with 500 mM NaCl; fractions were then separated on SDS/7.5% polyacrylamide gels and immunoblotted for γ-tubulin (γ), α-tubulin (α) or heat-shock protein Hsp70 (hp). Calibration standards (in kDa) are indicated on the top. Numbers at the bottom denote individual fractions.

Size distribution of γ-tubulin in high-speed extracts was assessed by gradient centrifugation. Immunoblotting experiments with separated fractions revealed that γ-tubulin was distributed in a large zone, and was present in complexes of various sizes (Figure 2, panel labelled 'γ'). Complexes of various sizes were also found for α-tubulin; however, the distribution pattern was different, and lower-molecular-mass complexes were found to prevail there (Figure 2, panel labelled 'α'). The distribution pattern of β-tubulin was similar to that of α-tubulin (results not shown). On the other hand, the heat-shock protein Hsp70 was absent in high-molecular-mass complexes, indicating that not all proteins had the same size distribution under the separation conditions used (Figure 2, panel labelled 'hp'). Large complexes of γ-tubulin were disrupted in samples containing 500 mM NaCl (Figure 2, panel labelled 'γ NaCl'). On the other hand, the same salt concentration had little effect on large complexes containing tubulin dimers (Figure 2, panel labelled 'α NaCl'). This indicates that brain γ-tubulin is present in high-molecular-mass complexes, which are susceptible to a change in salt concentration, and that these complexes differ in their properties from those that contain tubulin dimers.

When MTP-2 and purified tubulin were separated under non-denaturing conditions, multiple tubulin oligomers were detected in both samples, and BSA at the same protein concentration ran primarily as a single band (Figure 3A). The presence of microtubule-associated proteins in MTP-2 was without an observable effect on the mobility of tubulin oligomers, as revealed by the staining with anti-(α-tubulin) antibody (Figure 3B) or anti-(β-tubulin) antibody (results not shown). Multiple resolvable bands were also detected in MTP-2 using the antibody against γ-tubulin (Figure 3C). In contrast with the tubulin oligomers 'ladder', γ-tubulin was not detected at the position corresponding to the fastest migrating species of tubulin subunits, which are primarily monomers [37]. The fastest migrating band stained with anti-(γ-tubulin) antibody was in the position of putative αβ-tubulin heterodimers [37]; the other bands with lower mobilities were in positions different from those occupied by oligomers

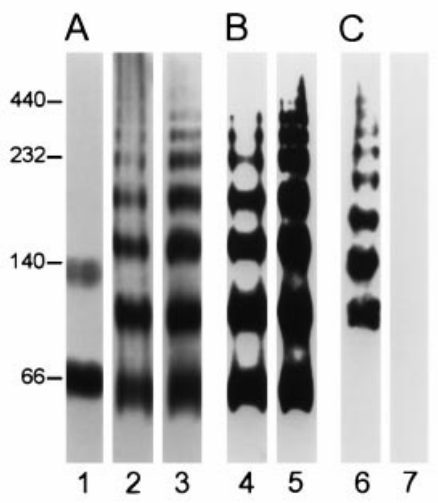

Figure 3 Immunoblot analysis of MTP and purified tubulin separated by native PAGE

BSA (lane 1), MTP-2 (lanes 2, 4 and 6) and tubulin (lanes 3, 5 and 7) were separated on non-denaturing 7% polyacrylamide gels. (**A**) Staining of transferred proteins; (**B**) immunostaining with anti-(α-tubulin) antibody TU-01; (**C**) immunostaining with anti-(γ-tubulin) antibody GTU-88. Aliquots of protein (3 μg) were loaded in lanes 1, 2, 4 and 6, and 5 μg aliquots of protein were loaded in lanes 3, 5 and 7. Molecular-mass markers (in kDa) are indicated on the left.

formed by $\alpha\beta$-tubulin dimers. These data indicate that γ-tubulin itself and/or γ-tubulin with another (unidentified) protein has the ability to form oligomers under conditions of native electrophoresis. The mobilities of these oligomers differ from those formed by $\alpha\beta$-tubulin heterodimers.

Association of γ-tubulin with $\alpha\beta$-tubulin dimers

Immunoprecipitation experiments showed that γ-tubulin could be precipitated specifically from high-speed extracts with monoclonal antibody TU-31 (IgG2b) (Figure 4A, lane 2). When the immobilized antibody was incubated without extract, no staining in the position of γ-tubulin was observed (Figure 4A, lane 3). No binding of γ-tubulin to immobilized Protein A was detected (Figure 4A, lane 4), and the control antibody NF-09 (IgG2b) produced no precipitation of γ-tubulin (results not shown). The possibility of an unspecific attachment of cytoplasmic proteins to possible γ-tubulin complexes was obviated, since no Hsp70 was detectable in the immunoprecipitated material with anti-Hsp70 antibody (Figure 4B, lane 2). Neither was there any co-precipitation of actin when the material was probed with anti-actin antibody (results not shown). γ-Tubulin precipitation was also observed with a low amount of purified antibody TU-31 directly bound covalently to Sepharose 4B (Figure 4C, lane 2). Probing of the precipitated material with anti-tubulin antibody revealed the presence of α-tubulin (Figure 4D, lane 2). The band corresponding to α-tubulin was discernible just under the band pertaining to the immobilized TU-31 antibody (marked with an arrow in Figure 4), so that it could potentially reflect a proteolytic fragmentation of the antibody. When the immobilized antibody alone was incubated without the extract, no band in this position was detected, therefore excluding the presence of a proteolytic fragment of antibody (Figure 4D, lane 3). Moreover, no band in this position was detected when the blot was probed with anti-(γ-tubulin) antibody (Figure 4C, lane 2). The reactivity with a protein in the position of α-tubulin was clearly recognized only when a less sensitive method of immunoglobulin detection with chromogenic substrate for alkaline phosphatase was applied.

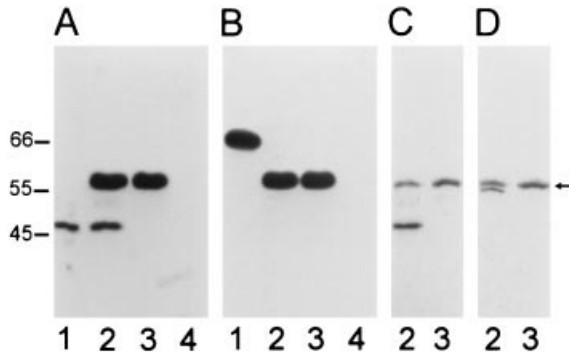

Figure 4 Immunoprecipitation of pig brain extract with anti-(γ-tubulin) antibody

Samples were precipitated with antibody TU-31 (IgG2b) bound to immobilized Protein A (**A**, **B**), or TU-31 covalently bound to Sepharose 4B (**C**, **D**). Proteins remaining after precipitation (lane 1), immunoprecipitated proteins (lane 2), immobilized immunoglobulins not incubated with cell extract (lane 3) and proteins bound to Protein A without antibody (lane 4) were separated on SDS/7.5% polyacrylamide gels. (**A** and **C**) Immunostaining with anti-(γ-tubulin) antibody GTU-88. (**B**) Immunostaining with control antibody raised against heat-shock protein Hsp70. (**D**) Immunostaining with anti-(α-tubulin) antibody TU-01. Bound antibodies were detected by chemiluminescent reagents (**A**, **B**) or by chromogenic substrates for alkaline phosphatase (**C**, **D**). Molecular-mass markers (in kDa) are indicated to the left of (**A**). The arrow on the right indicates the position of heavy chains of IgG.

During chemiluminescent detection, the two closely spaced bands appeared on the film as a single broad band.

Since the described experiments indicated that tubulin dimers could be present in γ-tubulin complexes, immunoprecipitation was also performed with the immobilized anti-(α-tubulin) antibody, TU-16 (IgM). This antibody specifically precipitated α-tubulin (Figure 5A, lane 2), and no binding of α-tubulin to immobilized Protein L was detected (Figure 5A, lane 4). Probing of the immunoprecipitated material with anti-(β-tubulin) antibody TU-06 confirmed the presence of β-tubulin subunits (results not shown). Probing of the immunoprecipitated material with anti-(γ-tubulin) antibody revealed that the precipitated $\alpha\beta$-tubulin dimers also contained γ-tubulin (Figure 5B, lane 2). When the immobilized antibody was incubated without extract, no staining in the position of γ-tubulin was observed (Figure 5B, lane 3). Control antibody VI-01 (IgM) did not elicit precipitation of γ-tubulin (results not shown). To test the possibility that cytoplasmic proteins might be unspecifically attached to the complexes containing $\alpha\beta$-tubulin dimers, the immunoprecipitated material was probed with anti-actin antibody, but no actin was detected (Figure 5C, lane 2). Similarly, probing with anti-Hsp70 antibody showed no co-precipitation of the relevant protein (results not shown). When the pooled fractions from gradient centrifugation corresponding to γ-tubulin complexes of low- (Figure 2, fractions 3 and 4) and high- (Figure 2, fractions 8 and 9) molecular mass were used for precipitation with TU-16 antibody, a large amount of tubulin dimers was precipitated from the low-molecular-mass fraction, whereas a substantially lower amount was precipitated from high-molecular-mass fractions. γ-Tubulin was specifically co-precipitated in both cases, but the yield was again higher with low-molecular-mass fractions. γ-Tubulin was also co-precipitated from fraction 2 (Figure 2). This suggests that tubulin dimers can interact with γ-tubulin irrespective of the size of complexes.

γ-Tubulin isoforms

To compare the electrophoretic behaviour of $\alpha\beta$-tubulin dimers and γ-tubulin, we have used different types of SDS for denatur-

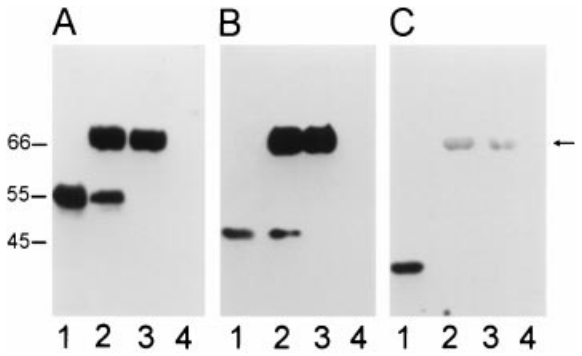

Figure 5 Immunoprecipitation of pig brain extract with anti-(α-tubulin) antibody

Samples were precipitated with antibody TU-16 (IgM) bound to immobilized Protein L. Proteins remaining after precipitation (lane 1), immunoprecipitated proteins (lane 2), immobilized immunoglobulin not incubated with cell extract (lane 3) and proteins bound to Protein L without antibody (lane 4) were separated on SDS/7.5% polyacrylamide gels. (**A**) Immunostaining with anti-(α-tubulin) antibody TU-01. (**B**) Immunostaining with anti-(γ-tubulin) antibody GTU-88. (**C**) Immunostaining with control anti-actin antibody. Molecular-mass markers (in kDa) are indicated on the left of (**A**). The arrow on the right indicates the position of heavy chains of IgM.

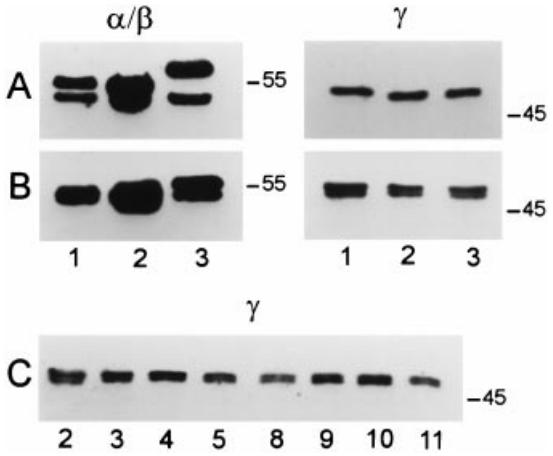

Figure 6 Influence of SDS-composition on electrophoretic properties of γ-tubulin

High-speed supernatant of brain extract (lane 1), MTP-2 (lane 2) and carboxyamidomethylated MTP-2 (lane 3) were separated on SDS/7.5% polyacrylamide gels using (**A**) 66%-purity SDS or (**B**) 99%-purity SDS. 'α/β' denotes immunostaining with a mixture of antibodies TU-01 and TU-06 against α- and β-tubulin respectively, 'γ' denotes immunostaining with anti-(γ-tubulin) antibody TU-32. (**C**) Sucrose-gradient centrifugation fractions separated on SDS/7.5% polyacrylamide gels in the presence of 99% SDS, immunostained with anti-(γ-tubulin) antibody GTU-88. Lanes 2–5 and 8–11 represent fractions shown in Figure 2. Molecular-mass markers (in kDa) are indicated on the right.

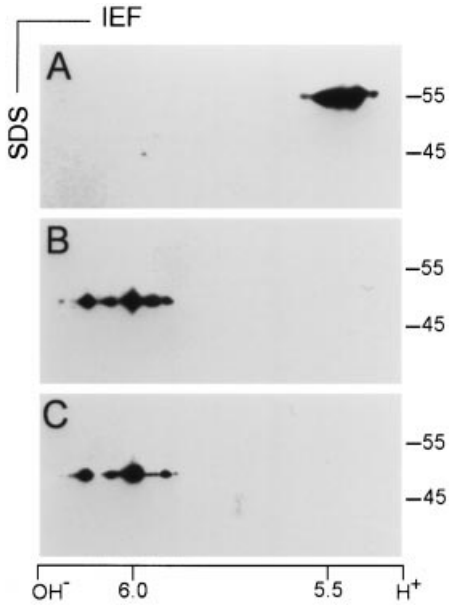

Figure 7 Immunoblot analysis of pig brain extract (A and B) and MTP-2 (C) separated by two-dimensional electrophoresis

Immunostaining with antibodies against α-tubulin (**A**) and γ-tubulin (**B**, **C**). The basic and acidic ends in the first dimension are marked by minus and plus signs respectively. The immunoblots depict the same region in the first dimension. Molecular-mass markers (in kDa) are indicated on the right. The pI scale is shown along the bottom of the Figure. IEF, isoelectric focusing.

ation and gel electrophoresis. For separation of tubulin dimers, we have routinely used lower-grade SDS, from Sigma, that contains hexadecyl and tetradecyl sulphate contaminants [34]. Under such conditions, clear two bands of tubulin dimers could be recognized in blots from a high-speed extract (Figure 6, lane 1), MTP-2 (lane 2) or carboxyamidomethylated MTP-2 (lane 3), where the separation between tubulin subunits was prominent (Figure 6A, panel labelled 'α/β'). On the other hand, in the same samples γ-tubulin provided one band (Figure 6A, panel labelled 'γ'). When high-grade SDS was used for the preparation of samples and following electrophoresis, α- and β-tubulin subunits were not separated in either high-speed extracts or in MTP-2. In carboxyamidomethylated MTP-2, the separation of tubulin subunits was easily distinguishable, but less prominently so (Figure 6B, panel labelled 'α/β'). However, γ-tubulin provided two bands in all samples tested (Figure 6B, panel labelled 'γ'). Carboxymethylation was without any effect on separation of the two γ-tubulin bands. The staining of two γ-tubulins was inhibited after pre-incubation of TU-32 antibody with the peptide used for immunization, but not with a control peptide from the N-terminal region of γ-tubulin. Two γ-tubulins were also detected with antibody GTU-88 against γ-tubulin amino acid region 38–53 (Figure 6C). These experiments eliminated the possibility that the reactivity was with a polypeptide of a similar electrophoretical mobility, but unrelated to γ-tubulin. The double band of γ-tubulins was easily recognizable when the detection method made use of a chromogenic substrate for alkaline phosphatase. With the chemiluminescence detection method, the two closely spaced bands were not so easily discernible. When high-grade SDS (99%) was used in SDS/PAGE during the separation of fractions from gradient centrifugation, doubling of the γ-tubulin band was observed in complexes of both low- and high-molecular mass (Figure 6C). Similarly, under the same conditions, a double γ-tubulin band was also detected in precipitates with anti-(γ-tubulin) and anti-(α-tubulin) antibodies.

The two-dimensional analysis revealed that soluble forms of γ-tubulin existed in multiple charge variants for which isoelectric points were more basic in comparison with α-tubulin isoforms (Figure 7A). At least five isoelectric variants of γ-tubulin were detected by immunoblotting in the high-speed extract (Figure 7B). The relative position of γ-tubulin isoforms with respect

to α-tubulin, and the number of γ-tubulin charge variants, in high-speed extracts were similar to those in MTP-2 (Figure 7C). The pI of the major γ-tubulin isoform was 6.00.

DISCUSSION

Fractionation of high-speed pig brain extracts by gradient centrifugation revealed the presence of γ-tubulin in complexes of different molecular masses. Large complexes dissociated in the presence of a high-salt concentration. The αβ-tubulin dimers were also distributed in a wide range of molecular masses. In the latter case, however, high-salt concentration did not induce a dissociation of large complexes. Non-denaturing PAGE of MTP-2 revealed disparate mobilities of oligomers containing αβ-tubulin dimers and oligomers containing γ-tubulin. In spite of that, γ-tubulin has the ability to interact with αβ-tubulin dimers in brain extracts. This was confirmed by partial cycling of γ-tubulin with tubulin dimers during repeated cycles of polymerization and depolymerization, as shown by Détraves et al. [9] and confirmed in the present study, and by our immunoprecipitation experiments. Our previous failure to detect γ-tubulin in pig brain MTP preparations with monoclonal antibodies [23] can most probably be ascribed to low titres of the used antibody batches.

The co-precipitation of αβ-tubulin dimers and γ-tubulin effected by monoclonal anti-(γ-tubulin) antibody, and co-precipitation of γ-tubulin and αβ-tubulin dimers effected by monoclonal anti-(α-tubulin) antibody, confirmed independently an association of soluble γ-tubulin with αβ-tubulin dimers in cold pig brain high-speed extracts. Multiple control tests have proved that the association is specific, and does not merely reflect a binding of abundant cytoplasmic proteins. The GTU-88 antibody directed against the amino acid region γ38–53 failed to precipitate γ-tubulin. This suggests that this region in brain γ-tubulin is not accessible for binding the antibody, in contrast with the γ434–449 region recognized by TU-31. However, it was reported that GTU-88 was effective in specific precipitation of γ-tubulin from leopard frog (*Rana pipiens*) oocytes [16]. This indicates that this γ-tubulin epitope is exposed differentially in separate model systems.

There are conflicting reports on the presence of αβ-tubulin heterodimers in γTuRC. Variable amounts of αβ-tubulin dimers have been found to co-precipitate with γ-tubulin in preparations from *Xenopus* oocytes [5], *Rana* oocytes [16], sheep brain [9] and cells of the lymphoblastic cell line KE37 [8]. No evidence of the presence of αβ-tubulin heterodimers in γTuRCs could be inferred from other studies [15,38]. One possible explanation of such discrepancies is that the interaction of γ-tubulin with αβ-tubulin dimers in cells is weak, and can be easily modified by sample preparation or by procedures used for the assessment of the composition of γ-tubulin complexes. Alternatively, γ-tubulin can be present in cells in other forms, except for γTuSC and γTuRC, which interact with tubulin dimers. γ-Tubulin dimer was identified under natural conditions in HeLa cells [39]. However, the significance of γ-tubulin interaction with αβ-tubulin dimers outside of γTuRCs remains to be elucidated. Experiments using *in vitro*-translated monomeric γ-tubulin showed that a single γ-tubulin subunit was sufficient to nucleate pig brain microtubules [40]. γ-Tubulin might also stabilize nucleation-competent stable oligomers formed by αβ-tubulin dimers or other types of tubulin oligomers. The capability of tubulin molecules to form various types of oligomers is widely recognized, and these oligomers have been assigned the role of regulators in microtubule dynamics [41].

It appeared relevant in this context to find out whether it would be possible to discriminate between γ-tubulin variants differing in their electrophoretic behaviour. To this end, the effect of SDS purity on the electrophoretic mobility of γ-tubulin was assessed in MTP and carboxyamidomethylated MTP. Although the hexadecyl and tetradecyl sulphate contaminants had a substantial effect on the mobility of α-tubulin subunit, as shown previously [26,34], they were without any effect on γ-tubulin mobility. On the other hand, SDS free of the contaminants led to a reproducible dissociation of γ-tubulin into its slow- and fast-migrating forms. Both polypeptides were detected by antibodies TU-32 and GTU-88 recognizing an epitope in the C-terminal and the N-terminal regions, respectively, of γ-tubulin molecule. The two isoforms were not drawn apart by gradient centrifugation, by cycling of MTP and by immunoprecipitation with anti-(γ-tubulin) or anti-(α-tubulin) antibodies. Two γ-tubulin isoforms were also detected in extracts from adult mouse brain (T. Sulimenko and P. Dráber, unpublished work). Although electrophoretic variants of γ-tubulin that can be discriminated by one-dimensional electrophoresis were not detected up to now in mammalian cells, fast- and slow-migrating γ-tubulin isoforms were distinguished in plants [42], *Physarum* [43] and *Drosophila* [44]. In *Drosophila*, they represent diverse gene products that are differentially expressed during development [45], and a loss of their function results in distinct phenotypes [46]. Two functional γ-tubulin genes were identified in mammals [47]. The observed fast- and slow-migrating γ-tubulin isoforms could represent the relevant proteins. Although both gene products differ in humans only in 12 amino acids [47], one cannot exclude a differential binding of SDS to polypeptides. Alternatively, cell heterogeneity in brain tissue or post-translational modification(s) could be responsible for the observed differences in mobility.

Neuronal α- and β-tubulin subunits are characterized by their high-charge heterogeneity, which stems not only from multiple gene expression, but also from numerous post-translational modifications [48]. Substantial changes in the number of tubulin isoforms were observed during brain development [49,50]. We have detected multiple charge variants of γ-tubulin in brain extracts, as well as in MTP-2. At least five clearly distinguishable isoelectric variants were identified in pig brain extract. This exceeds the number of functional γ-tubulin genes, and points to post-translational modification of γ-tubulin. Two major and two minor γ-tubulin isoforms were recognized by two-dimensional electrophoresis in sheep brain [9]. Phosphorylation of the γ-tubulin residue Tyr^{445}, which is invariant in all γ-tubulins and regulates the microtubule organization in budding yeast, has been reported recently [22]. γ-Tubulin was found in mouse mammary carcinoma cells in association with serine/threonine 'polo-like' kinase [51] and in rat basophilic leukaemia cells in complexes containing protein tyrosine kinase $p53/p56^{lyn}$ [52]. It is possible that phosphorylation or other post-translational modifications of neuronal γ-tubulin modify its interaction with tubulin dimers or other associated proteins.

In conclusion, the presented data demonstrate for the first time that there exist in brain two γ-tubulin isoforms, which persist in differently sized complexes and co-polymerize with tubulin dimers. An interaction of γ-tubulins with tubulin heterodimers irrespective of the size of γ-tubulin complexes reflects the presence of other γ-tubulin forms apart from γTuSC and γTuRC. Brain γ-tubulin is post-translationally modified, and these modifications possibly have an important role in the regulation of microtubule nucleation.

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REFERENCES

1 Kellogg, D. R., Moritz, M. and Alberts, B. M. (1994) The centrosome and cellular organization. Annu. Rev. Biochem. **63**, 639–674
2 Oakley, C. E. and Oakley, B. R. (1989) Identification of γ-tubulin, a new member of the tubulin superfamily encoded by *mip*A gene of *Aspergillus nidulans*. Nature (London) **338**, 662–664
3 Oakley, B. R., Oakley, C. E., Yoon, Y. and Jung, M. (1990) γ-Tubulin is a component of the spindle pole body that is essential for microtubule function in *Aspergillus nidulans*. Cell (Cambridge, Mass.) **61**, 1289–1301
4 Joshi, H. C., Palacios, M. J., McNamara, L. and Cleveland, D. W. (1992) γ-Tubulin is a centrosomal protein required for cell cycle-dependent microtubule nucleation. Nature (London) **356**, 80–83
5 Zheng, Y., Wong, M. L., Alberts, B. and Mitchison, T. (1995) Nucleation of microtubule assembly by a γ-tubulin-containing ring complex. Nature (London) **378**, 578–583
6 Moritz, M., Braunfeld, M. B., Fung, J. C., Sedat, J. W., Alberts, B. M. and Agard, D. A. (1995) Three-dimensional structural characterization of centrosomes from *Drosophila* embryos. J. Cell Biol. **130**, 1149–1159
7 Meads, T. and Schroer, T. A. (1995) Polarity and nucleation of microtubules in polarized epithelial cells. Cell Motil. Cytoskeleton **32**, 273–288
8 Moudjou, M., Bordes, N., Paintrand, M. and Bornens, M. (1996) γ-Tubulin in mammalian cells: the centrosomal and the cytosolic forms. J. Cell Sci. **109**, 875–887
9 Détraves, C., Mazarguil, H., Lajoie-Mazenc, I., Julian, M., Raynaud-Messina, B. and Wright, M. (1997) Protein complexes containing γ-tubulin are present in mammalian brain microtubule protein preparations. Cell Motil. Cytoskeleton **36**, 179–189
10 Moritz, M., Zheng, Y., Alberts, B. M. and Oegema, K. (1998) Recruitment of the γ-tubulin ring complex to *Drosophila* salt-stripped centrosome scaffolds. J. Cell Biol. **142**, 775–786
11 Murphy, S. M., Urbani, L. and Stearns, T. (1998) The mammalian γ-tubulin complex contains homologues of the yeast spindle pole body components Spc97p and Spc98p. J. Cell Biol. **141**, 663–674
12 Oegema, K., Wiese, C., Martin, O. C., Milligan, R. A., Iwamatsu, A., Mitchison, T. J. and Zheng, Y. (1999) Characterization of two related *Drosophila* γ-tubulin complexes that differ in their ability to nucleate microtubules. J. Cell Biol. **144**, 721–733
13 Knop, M. and Schiebel, E. (1997) Spc98p and Spc97p of the yeast γ-tubulin complex mediate binding to the spindle pole via their interaction with Spc110p. EMBO J. **16**, 6985–6995
14 Wiese, C. and Zheng, Y. (2000) A new function for the γ-tubulin ring complex as a microtubule minus-end cap. Nat. Cell Biol. **2**, 358–364
15 Wiese, C. and Zheng, Y. (1999) γ-Tubulin complexes and their interaction with microtubule-organizing centers. Curr. Opin. Struct. Biol. **9**, 250–259
16 Lessman, C. A. and Kim, H. (2001) Soluble tubulin complexes in oocytes of the common leopard frog, *Rana pipiens*, contain γ-tubulin. Mol. Reprod. Dev. **60**, 128–136
17 Li, Q. and Joshi, H. C. (1995) γ-Tubulin is a minus end-specific microtubule binding protein. J. Cell Biol. **131**, 207–214
18 Llanos, R., Chevrier, V., Ronjat, M., Meurer-Grob, P., Martinez, P., Frank, R., Bornens, M., Wade, R. H., Wehland, J. and Job, D. (1999) Tubulin binding sites on γ-tubulin: identification and molecular characterization. Biochemistry **38**, 15712–15720
19 Inclán, V. M. and Nogales, E. (2000) Structural models for the self-assembly and microtubule interactions of γ-, δ- and ε-tubulin. J. Cell Sci. **114**, 413–422
20 Liu, B., Marc, J., Joshi, H. C. and Palevitz, B. A. (1993) γ-Tubulin-related protein associated with microtubule arrays of higher plants in cell cycle-dependent manner. J. Cell Sci. **104**, 1217–1228
21 Binarová, P., Hause, B., Doležel, J. and Dráber, P. (1998) Association of γ-tubulin with kinetochore/centromeric region of plant chromosomes. Plant J. **14**, 751–757
22 Vogel, J., Drapkin, B., Oomen, J., Beach, D., Bloom, K. and Snyder, M. (2001) Phosphorylation of γ-tubulin regulates microtubule organization in budding yeast. Dev. Cell **1**, 621–631
23 Nováková, M., Dráberová, E., Schürmann, W., Czihak, G., Viklický, V. and Dráber, P. (1996) γ-Tubulin redistribution in taxol-treated mitotic cells probed by monoclonal antibodies. Cell Motil. Cytoskeleton **33**, 38–51
24 Viklický, V., Dráber, P., Hašek, J. and Bártek, J. (1982) Production and characterization of a monoclonal antitubulin antibody. Cell Biol. Int. Rep. **6**, 725–731
25 Dráberová, E. and Dráber, P. (1998) Novel monoclonal antibodies TU-08 and TU-16 specific for tubulin subunits. Folia Biol. (Prague) **44**, 35–36
26 Dráber, P., Dráberová, E., Linhartová, I. and Viklický, V. (1989) Differences in the exposure of C- and N-terminal tubulin domains in cytoplasmic microtubules detected with domain-specific monoclonal antibodies. J. Cell Sci. **92**, 519–528
27 Dráberová, E., Dráber, P., Havlíček, F. and Viklický, V. (1986) A common antigenic determinant of vimentin and desmin defined by monoclonal antibody. Folia Biol. (Prague) **32**, 295–303
28 Shelanski, M. L., Gaskin, F. and Cantor, C. R. (1973) Microtubule assembly in the absence of added nucleotides. Proc. Natl. Acad. Sci. U.S.A. **70**, 765–768
29 Mandelkow, E. M., Herrman, M. and Ruhl, V. (1985) Tubulin domains probed by limited proteolysis and subunit-specific antibodies. J. Mol. Biol. **185**, 311–327
30 Weingarten, M. D., Lockwood, A. H., Hwo, S. Y. and Kirschner, M. W. (1975) A protein factor essential for microtubule assembly. Proc. Natl. Acad. Sci. U.S.A. **72**, 1858–1868
31 George, H. J., Misra, L., Field, D. J. and Lee, J. C. (1981) Polymorphism of brain tubulin. Biochemistry **20**, 2402–2409
32 Dráberová, E. and Dráber, P. (1993) A microtubule-interacting protein involved in coalignment of vimentin intermediate filaments with microtubules. J. Cell Sci. **106**, 1263–1273
33 Dráber, P., Lagunowich, L. A., Dráberová, E., Viklický, V. and Damjanov, I. (1988) Heterogeneity of tubulin epitopes in mouse fetal tissues. Histochemistry **89**, 485–492
34 Best, D., Warr, P. J. and Gull, K. (1981) Influence of the composition of commercial sodium dodecyl sulfate preparations on the separation of α- and β-tubulin during polyacrylamide gel electrophoresis. Anal. Biochem. **114**, 281–284
35 Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature (London) **227**, 680–685
36 Görg, A., Obermaier, C., Boguth, G. and Weiss, W. (1999) Recent developments in two-dimensional gel electrophoresis with immobilized pH gradients: wide pH gradients up to pH 12, longer separation distances and simplified procedures. Electrophoresis **20**, 712–717
37 Correia, J. J. and Williams, Jr, R. C. (1985) Characterization of oligomers of tubulin by two-dimensional native electrophoresis. Arch. Biochem. Biophys. **239**, 120–129
38 Murphy, S. M., Preble, A. M., Patel, U. K., O'Connell, K. L., Dias, D. P., Moritz, M., Agard, D., Stults, J. T. and Stearns, T. (2001) GCP5 and GCP6: two new members of the human γ-tubulin complex. Mol. Biol. Cell **12**, 3340–3352
39 Vassilev, A., Kimble, M., Silflow, C. D., LaVoie, M. and Kuriyama, R. (1995) Identification of intrinsic dimer and overexpressed monomeric forms of γ-tubulin in Sf9 cells infected with baculovirus containing the *Chlamydomonas* γ-tubulin sequence. J. Cell Sci. **108**, 1083–1092
40 Leguy, R., Melki, R., Pantaloni, D. and Carlier, M. F. (2000) Monomeric γ-tubulin nucleates microtubules. J. Biol. Chem. **275**, 21975–21980
41 Caudron, N., Valiron, O., Usson, Y., Valiron, P. and Job, D. (2000) A reassessment of the factors affecting microtubule assembly and disassembly *in vitro*. J. Mol. Biol. **297**, 211–220
42 Petitprez, M., Caumont, C., Barthou, H., Wright, M. and Alibert, G. (2001) Two γ-tubulin isoforms are differentially expressed during development in *Helianthus annuus*. Physiol. Plant. **111**, 102–107
43 Lajoie-Mazenc, I., Détraves, C., Rotaru, V., Gares, M., Tollon, Y., Jean, C., Julian, M., Wright, M. and Raynaud-Messina, B. (1996) A single γ-tubulin gene and mRNA, but two γ-tubulin polypeptides differing by their binding to the spindle pole organizing centres. J. Cell Sci. **109**, 2483–2492
44 Raynaud-Messina, B., Debec, A., Tollon, Y., Gares, M. and Wright, M. (2001) Differential properties of the two *Drosophila* γ-tubulin isotypes. Eur. J. Cell Biol. **80**, 643–649
45 Wilson, P. G., Zheng, Y., Oakley, C. E., Oakley, B. R., Borisy, G. G. and Fuller, M. T. (1997) Differential expression of two γ-tubulin isoforms during gametogenesis and development in *Drosophila*. Dev. Biol. **184**, 207–221
46 Llamazares, S., Tavosanis, G. and Gonzalez, C. (1999) Cytological characterisation of the mutant phenotypes produced during early embryogenesis by null and loss-of-function alleles of the *γTub37C* gene in *Drosophila*. J. Cell Sci. **112**, 659–667
47 Wise, D. O., Krahe, R. and Oakley, B. R. (2000) The γ-tubulin gene family in humans. Genomics **67**, 164–170
48 Luduena, R. F. (1998) Multiple forms of tubulin: different gene products and covalent modifications. Int. Rev. Cytol. **178**, 207–275
49 Wolff, A., Denoulet, P. and Jeantet, C. (1982) High level of tubulin microheterogeneity in the mouse brain. Neurosci. Lett. **31**, 323–328
50 Linhartová, I., Dráber, P., Dráberová, E. and Viklický, V. (1992) Immunological discrimination of β-tubulin isoforms in developing mouse brain. Posttranslational modification of non-class III β-tubulins. Biochem. J. **288**, 919–924
51 Feng, Y., Hodge, D. R., Palmieri, G., Chase, D. L., Longo, D. L. and Ferris, D. K. (1999) Association of polo-like kinase with α-, β- and γ-tubulins in a stable complex. Biochem. J. **339**, 435–442
52 Dráberová, L., Dráberová, E., Surviladze, Z., Dráber, P. and Dráber, Pa. (1999) Protein tyrosine kinase $p53/p56^{lyn}$ forms complexes with γ-tubulin in rat basophilic leukemia cells. Int. Immunol. **11**, 1829–1839

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