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γ-Tubulin in mammalian cells: the centrosomal and the cytosolic forms

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SUMMARY

The centrosome is one of the cellular organelles for which the mechanism by which it operates still remains to be unravelled. The finding of the association with the centrosome of γ-tubulin, a protein which belongs to the tubulin superfamily, has provided a long sought after biochemical tool with which to address centrosome function. We have generated a specific anti-γ-tubulin polyclonal antibody to study the biochemical properties and the cellular distribution of the human lymphoblastic γ-tubulin. Using cell fractionation and mass isolation of centrosomes, we observed that in contrast to the figures suggested by immunofluorescence, a minimum figure of 80% of total γ-tubulin exists as a cytosolic form. The centrosomal form, for which at least half is not strongly associated with the centrosome, behaves in two-dimensional gel electrophoresis identically to the soluble form (as at least two spots of a pI of around 6). Post-embedding immunolocalization reveals that γ-tubulin is distributed in the pericentriolar matrix but is also closely associated with centrioles. Using a combination of gel filtration, ion exchange chromatography, equilibrium sucrose gradient centrifugation and immunoprecipitation, we show that the major part of cytosolic γ-tubulin might be involved in complexes heavier than the Tcp1 particle. We further demonstrate, by co-immunoprecipitation of γ-tubulin and Tcp1 with either anti-Tcp1 or anti-γ-tubulin antibodies, that a small part of γ-tubulin participates in Tcp1-γ-tubulin particles. Interestingly, the soluble form of γ-tubulin co-purifies with taxol-stabilized microtubules and its association with microtubules resisted salt, ATP and GTP treatments. The existence of a centrosomal form and a large pool of cytosolic γ-tubulin-containing complexes in somatic cells suggests that the overall γ-tubulin cellular distribution does not seem to be as straightforward as it was drawn earlier.

Key words: γ-Tubulin, Centrosome, Cytosol, Chaperone, HSP, Human cell

INTRODUCTION

The temporal and spatial distribution of the interphase and mitotic microtubule (MT) networks are determined to a large extent by the activities of the centrosome (Kimble and Kuriyama, 1992) and, consequently, this organelle may play a crucial pivotal role in the determination of the overall cytoplasmic organization, polarity and fidelity of transmission of the genetic material during cell division (Tournier and Bornens, 1994). In animal cells, the centrosome is composed of two centrioles, one of which possesses two sets of appendages, surrounded by centrosomal matrix (Paintrand et al., 1992). Despite numerous investigations aimed at a biochemical dissection and genetic analysis of the centrosome, or of its functional equivalents (Kalt and Schliwa, 1993; Rout and Kilmartin, 1990; Kilmartin, 1994; Kuriyama, 1992; Page et al., 1994; Raff et al., 1993), little is known of the molecular mechanisms which govern its main functions: the nucleation of MTs and its precise assembly during the controlled cycle of its duplication. The recent decade has, however, seen an increase in our understanding of the relationships between certain centrosomal components and their potential roles in the function of this organelle (Doxsey et al., 1994; Moudjou et al., 1991; Oakley et al., 1990; Spang et al., 1993, 1995; Winey et al., 1991). Among the identified centrosomal proteins, γ-tubulin (Oakley, 1992) has stimulated great interest, since one of the established models for MT-nucleating activity suggested that 'the active principle of the centrosome might be a 'seed' in which some components (perhaps including tubulin) set up a geometry sufficiently similar to the end of a microtubule that the initiation event is energetically similar to the elongation reaction' (from McIntosh, 1983).

The γ-tubulin gene, first identified in *Aspergillus nidulans* (Oakley and Oakley, 1989; Oakley et al., 1990), has been shown to encode a protein which is located at the spindle pole body of *A. nidulans*, and has been now identified and located at the centrosome in a wide variety of cells (Stearns et al., 1991; Zheng et al., 1991). Moreover, γ-tubulin was also localized in acentriolar nucleating centers during early mouse development (Gueth-Hallonet et al., 1993) and in higher plant cells (Liu et al., 1993), and in the vicinity of basal bodies in both bovine retinal photoreceptors and epithelial ciliated cells (Muresan et al., 1993). Anti-γ-tubulin antibodies inhibit MT regrowth from the centrosome in permeabilized mammalian

cells (Joshi et al., 1992), suggesting that γ-tubulin might be a component of the MT-nucleating complex throughout the cell cycle (Oakley, 1992; Felix et al., 1994; Stearns and Kirschner 1994). Nevertheless, it is clearly established that in some situations, the presence of γ-tubulin is not systematically indicative of a MT-nucleating activity. For example, the presence of γ-tubulin is not sufficient either to activate immature frog sperm centrosomes incubated in egg extracts containing anti-pericentrin antibodies (Doxsey et al., 1994), or to confer a MT-nucleating activity on interphase *Schizosaccharomyces pombe* spindle pole bodies (Masuda et al., 1992). These results suggest that other requirements are necessary for a centrosome to be competent for MT-nucleation (Archer and Solomon, 1994).

In addition to the spindle pole body, γ-tubulin has been also localized at the cell equatorial putative MT-nucleating centers, which appear during telophase and cytokinesis in *Schizosaccharomyces pombe* (Horio et al., 1991; Hagan and Hyams 1988). This result is reminiscent of the finding that in PtK_2 cells, γ-tubulin accumulates on MTs close to the spindle poles during metaphase (Lajoie-Mazenec et al., 1994) and transiently at the minus end of the two interdigitating MT bundles constituting the midbody in late telophase (Julian et al., 1993). Interestingly, microinjection of γ-tubulin antibodies during anaphase prevents the subsequent formation of the midbody between two separating cells. This result suggests that the MTs constituting the midbody may be nucleated from special sites, which are assembled or become active during late telophase (Julian et al., 1993).

More recently, Felix et al. (1994) and Stearns and Kirschner (1994) Have both described the existence of γ-tubulin in a soluble form in *Xenopus* egg extracts. This soluble form was shown to be associated with a large complex capable of binding to DMSO-stabilized MTs in *Xenopus* eggs (25 S γ-some; Stearns and Kirschner, 1994), and to be involved in the maturation process of inactive sperm centrioles (Felix et al., 1994; Stearns and Kirschner, 1994). However, components of this complex have yet to be identified. In addition, a γ-tubulin monomer form was observed under high salt conditions after overexpression of *Chlamydomonas* γ-tubulin in insect Sf9 cells, whilst the endogenous γ-tubulin was mainly involved in a dimer which was not further characterized (Vassiliev et al., 1995). The partially purified monomeric form of *Chlamydomonas* γ-tubulin was shown to also associate with MTs in a salt dependent manner (Vassiliev et al., 1995).

In the present work, we have attempted to clarify the cellular distribution of γ-tubulin in human lymphoblastic cells by comparing its centrosomal and cytosolic forms. We have carried out a biochemical characterization of γ-tubulin association with the centrosome, and proteins with which cytosolic γ-tubulin could interact. We also provide a detailed ultrastructural study, using a post embedding approach, of the localization of γ-tubulin within the centrosome.

MATERIALS AND METHODS

Cell culture

The human lymphoblastic KE37 cell line was cultured in suspension in RPMI 1640 medium (Eurobio laboratories) supplemented with 10% foetal calf serum (FCS) at 37°C and cultured in an environment with 5% CO2 in air. HeLa cells were cultured in MEM medium containing 10% FCS.

Antibodies

For anti-γ-tubulin polyclonal antibody generation, the γ-tubulin peptide 38-53 EEFATEGTDRKDVFFY-C was synthesized. This synthetic peptide was coupled through the C-terminal cysteine to maleimide-activated thyroglobulin (Sigma). The maleimide-activated thyroglobulin was obtained with sulfo-MBS (Pierce, Rockford). After emulsification with complete Freund's adjuvant (v/v), 500 μg of the immunogen was injected subcutaneously into three rabbits. Booster injections were given after three weeks and subsequently every ten days for 9 times with 200 μg of the immunogen diluted with incomplete Freund's adjuvant at a ratio of 1:1. Sera were screened by immunoblotting against centrosomal proteins and by immunofluorescence labelling of HeLa cells. IgGs from the best reacting serum were isolated by passing serum through a Protein A-Sepharose column (Pharmacia) and the IgGs specific for γ-tubulin were then subsequently immunoaffinity purified with the synthetic peptide coupled to BSA-Sepharose beads. Anti-γ-tubulin IgGs were eluted from the column at pH 2.8. However, pH 2.2 was necessary for their elution from the synthetic peptide coupled to BSA-Sepharose beads. This indicates that the antibody possesses a high affinity for this peptide.

Another polyclonal anti-γ-tubulin antibody was obtained from Dr B. R. Oakley (Ohio state university, Colombus, USA; Zheng et al., 1991). This antibody was used in the immunofluorescence experiment shown in Fig. 2. Monoclonal antibodies (mAbs) N356 and N357 specific for α- and β-tubulin, respectively, were purchased from Amersham. mAb GT335 (Wolff et al., 1992) directed against the C-terminal polyglutamylated α- and β-tubulins was a gift from B. Eddé (Collège de France Paris, France). This mAb was used for electron microscopy immunolocalization. Anti-Tcp1 rat monoclonal antibodies 91A and 84A (Willison et al., 1989) were a generous gift from Dr K. Willison (ICRF, London, UK). mAb H3F18 (Blisnick et al., 1988) recognizing antigens of the HSP70 family in a wide variety of cells was a generous gift from Dr P. Dubois (Institut Pasteur Paris, France). Polyclonal anti-HSP90 protein was kindly provided by Dr M. G. Catelli (INSERM U33 Cremlin Bicetre, France). CTR453 (Bailly et al., 1989; 1992) and CTR210 anti-centrosome mAbs (Fleury et al., 1993; Perret et al., 1995) were obtained after immunization of mouse with isolated human lymphoblastic centrosomes.

Cell fractionation

Isolation and extraction of centrosomes

Centrosomes were isolated from KE37 cells as previously described (Moudjou and Bornens, 1994). Extractions of isolated centrosomes by 0.5 M KI in 10 mM K Pipes, pH 7.2, 1D buffer (50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 0.25 M NaCl, 0.5% Nonidet P40) and by 3D buffer (100 mM Tris-HCl, pH 8.3, 2 mM EDTA, 0.5% deoxycholate, 0.5% Nonidet P40, 0.1% SDS) were performed as described by Moudjou et al. (1991). ATP extraction conditions were achieved by adding 10 mM ATP, 5 mM $MgSO_4$ and 1 mM DTT to centrosomes resuspended in 10 mM K Pipes, pH 7.2. Heat treatment of isolated centrosomes was performed in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA for 15 minutes at 45°C according to the method of Steffen et al. (1989).

Preparation of KE37 Triton X-100 soluble and insoluble proteins

Triton X-100 soluble and insoluble proteins were obtained as described by Moudjou et al. (1991).

Preparation of taxol-stabilized MTs from human cells

Taxol (Tx)-stabilized MTs were obtained from the cytosolic fractions of human lymphoblastic cultured cells according to the method described by Paschal et al. (1987). Taxol was used at a final concentration of 20 μM. AMP-PNP (1 mM; Sigma) and 5 units/ml of apyrase

(Sigma) were added in the MT polymerization step to reduce nucleotide levels in the extracts. The Tx-stabilized MTs thus obtained were sedimented onto a 5% sucrose (in PEM buffer) cushion and resuspended in PEM buffer (100 mM Pipes, pH 6.8, 2 mM EGTA, 1 mM $MgSO_4$, 1 mM PMSF and 1 μg/ml each of aprotinin, chymostatin and pepstatin) containing 5 μM taxol. These Tx-stabilized MTs were then treated with the following different reagents: 10 mM Mg-ATP, 1 mM DTT - 10 mM Mg-GTP, 1 mM DTT - NaCl 0.6 M. Following a 30 minute incubation at room temperature, the samples were centrifuged at 20,000 ***g*** for 20 minutes. Samples from the supernatant and pellet fractions were diluted in boiling SDS-PAGE sample buffer, whilst the remainder was immediately frozen with liquid nitrogen and stored at –80°C.

Preparation of the cytosolic fraction

Human KE37 cells were harvested and washed with TBS buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl). The pellet was frozen with liquid nitrogen, thawed rapidly and resuspended in 5-7 ml of cold TNM buffer (10 mM Tris-HCl, pH 7.2, 10 mM NaCl, 1 mM $MgCl_2$) containing a mixture of protease inhibitors (aprotinin, leupeptin and pepstatin at 1 μg/ml and benzamidine at 1 mM). The resuspended cells were disrupted in a Dounce homogenizer (15-20 strokes) at 4°C. All the subsequent steps were done at 4°C. The homogenate was immediately centrifuged at 150,000 ***g*** in a Beckman ultracentrifuge with an SW55 swinging bucket rotor for 75 minutes. Following this centrifugation, a thin layer of lipid was removed by aspiration from the top of the supernatant, and the latter (representing the cytosolic fraction) was recovered. This fraction could be used either immediately or frozen as aliquots with liquid nitrogen and stored at –80°C.

Gel exclusion chromatograpy

The cytosol fraction was applied to a 1 m × 0.5 cm Sephacryl S-300 gel filtration column (Pharmacia) equilibrated and run in TNM buffer at 4°C. Fractions (1 ml) were monitored by Coomassie staining of SDS-PAGE and by immunodetection with different antibodies after transfer to nitrocellulose. The high molecular mass fractions enriched in γ-tubulin were immediately loaded onto a 15-40% equilibrium sucrose gradient (see below).

Cation-exchange phosphocellulose P11 chromatography

A fraction of the cytosol (30-40 mg) obtained as described above was dialysed against the P11 column buffer (20 mM K Pipes, pH 6.8, 10 mM NaCl, 1 mM EDTA and 2 mM $MgCl_2$), clarified by a rapid centrifugation with 2-MK Sigma centrifuge at 20,000 ***g*** for 5 minutes and applied to the P11 column. After removal of unbound proteins (fraction 0) and column washing, bound proteins were eluted by either a linear salt gradient from 10 mM to 500 mM NaCl in P11 buffer or by one step using P11 buffer containing 0.5 M NaCl. This fraction was called the P11 fraction.

Equilibrium sucrose gradient centrifugation

The high molecular mass fraction obtained from the Sephacryl S-300 gel filtration column (or the P11 fraction) was applied to a 15-40% sucrose linear gradient (6 ml) in TNM buffer. Centrifugation was performed using a SW41 swinging bucket rotor at 100,000 ***g*** for 16 hours. After centrifugation, 20 fractions (400 μl each) were collected starting from the bottom of the gradient. A sample from each fraction was taken out and diluted with 8-fold concentrated SDS-PAGE sample buffer (without glycerol, 100°C, 5 minutes) to avoid excessive dilution of the samples, and the remainder of the fractions was stored at –80°C after freezing in liquid nitrogen.

Immunoprecipitation experiments

The P11 fraction or the fraction from the bottom of the sucrose gradient (which are enriched in γ-tubulin) were dialysed overnight against 1D buffer (see above). After a rapid centrifugation to eliminate trace aggregates, primary antibodies were added to the samples and the mixture incubated for 2 hours at 4°C. Protein A-Sepharose beads, equilibrated with 1D buffer, were added to each sample and left under mild agitation for 1 hour at 4°C. Protein A-Sepharose beads (Pharmacia) were then sedimented. A sample from the supernatants was denatured with SDS-PAGE sample buffer (100°C, 5 minutes). The sedimented Protein A-Sepharose beads were washed (5 times) with 500 μl of 1D buffer and with 1 ml of double distilled water for 3 times. The immunoprecipitates were solubilized from the Sepharose beads by incubation with the SDS-PAGE sample buffer (100°C, 5 minutes) and centrifugation.

Immunofluorescence microscopy

HeLa cells were first extracted with 0.5 % Triton X-100 in PHEM buffer before methanol fixation and processed for double immunofluorescence labelling experiments as described by Moudjou et al. (1991). We have used four different anti-γ-tubulin antibodies. Only pictures from the affinity purified anti-human γ-tubulin IgGs provided by B. R. Oakley are shown. Mixed rhodamine-conjugated goat anti-mouse and fluorescein labelled goat anti-rabbit (Jackson) were used as secondary antibodies.

Post-embedding immunolocalization on isolated centrosomes

Isolated centrosomes were sedimented onto glass coverslips as described previously (Moudjou et al., 1991) with slight modifications. Centrosome fixation was carried out for 1 minute in 1.25% glutaraldehyde in 0.1 M cacodylate buffer containing 4% LMGG and for 29 minutes in 0.125% glutaraldehyde in 0.1 M cacodylate buffer containing 4% LMGG and 4% sucrose, followed by one rinse in 0.1 M cacodylate buffer containing 4% sucrose and one rinse in K Pipes buffer. A 3× 10 minute neutralization was carried out with 1 mg/ml $NaBH_4$ in K Pipes buffer followed by a last rinse in the same buffer. After dehydration, in 50% ethanol (2× 5 minutes) and 70% ethanol (2× 5 minutes), centrosomes were embedded in a mixture of LR-White/ethanol at a ratio of 2:1 for 30 minutes, pure LR-White for 3 hours at room temperature with three changes, and polymerized for 48 hours at 54°C. The LR-White samples were dissociated from the coverslips by alternate hot/cold shocks using boiling water and liquid nitrogen. Sections parallel to the coverslips were placed onto nickel grids and used for the post embedding immunolocalization of centrosomal antigens and observed with a Philips EM 201 electron microscope after double contrast with uranyl acetate and lead citrate. Alternatively, sedimented centrosomes were prefixed according to the method of Burgess et al. (1991) and embedded in the hydrophile resin, Unicryl, according to the method of Scala et al. (1992) with one modification: polymerisation was carried out at 55°C for 48 hours instead of using UV light.

LR-White sections were incubated with the primary antibody in TBS (20 mM Tris, 150 mM NaCl), pH 7.2, containing 0.2% bovine serum albumin and 0.2% Tween-20, for 90 minutes at room temperature. Two 5 minute washes in the same buffer followed by two 5 minute washes in TBS, pH 8.2, containing 0.2% bovine serum albumin and 0.2% Tween-20 were carried out before the addition of the secondary antibody GAR 5 (Amersham France SA, Les Ulis, France) diluted 20-fold in TBS, pH 8.2, containing 0.2% bovine serum albumin and 0.2% Tween-20. Unicryl sections were processed identically, but a preliminary step was carried out in which sections were incubated for 10 minutes with an unlabelled goat serum diluted ten-fold in TBS, pH 7.2, containing 0.2% bovine serum albumin and 0.2% Tween-20.

Electrophoresis and immunoblotting

Analytical electrophoresis was performed using either 6-15% polyacrylamide gradient SDS-PAGE or SDS-PAGE of uniform concentration according to the method of Laemmli (1970). The molecular mass standards were: myosin heavy chain, 200 kDa; β-galactosidase, 116 kDa; phosphorylase b, 97 kDa; bovine serum albumin, 67 kDa;

ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 21 kDa; lysozyme, 14 kDa. Gels were stained with either Coomassie blue or using a silver nitrate staining method.

Two-dimensional (2-D) gel electrophoresis was achieved by separating the proteins in the first dimension by equilibrium pH gradient electrophoresis (IEF) as described by O'Farrell (1975). Disposable pre-calibrated 50 μl pipettes (Vitrex, ModulHom, Danemark) were used to prepare the first dimension (1-D) gels to enable their use on minigels for the second dimension.

Samples resolved on 1-D or 2-D gels were electrophoretically transferred onto nitrocellulose filters and processed for immunoblotting as described previously (Moudjou et al., 1991).

RESULTS

The cellular distribution of γ-tubulin

Polyclonal anti-γ-tubulin sera were raised against the 16 amino-terminal residues (38-53) of γ-tubulin (Joshi et al., 1992; Julian et al., 1993). We systematically used the affinity purified IgGs in all the subsequent experiments (see Material and Methods). Anti-γ-tubulin IgGs recognized a polypeptide with an apparent molecular mass of 46 kDa, which migrates faster than either α- and β-tubulin polypeptides (Fig. 1A, right panel, and 1C, lane ctr). No cross-reaction with either α- or β-tubulin was observed. A faint band (migrating around 67 kDa) was, however, detected in the Triton-insoluble fraction (Fig. 1A). Preincubation of the anti-γ-tubulin IgGs with the immunogenic peptide abolished the γ-tubulin detection in immunoblotting experiments.

When western immunoblotting using anti-γ-tubulin IgGs was carried out on Triton X-100-soluble and -insoluble fractions (40 μg protein each) and centrosomal proteins (0.6 μg protein) from KE37 human cells, results similar to those shown in Fig. 1A (right panel) were obtained: γ-tubulin was enriched (as judged from signal intensity) in the centrosomal fraction. Nevertheless, a significant signal was also detected in both Triton-soluble and -insoluble fractions. The γ-tubulin detected in the Triton-insoluble fraction could in part correspond to the centrosomal form as the centrosome is still associated with the nucleus in this fraction. This partition of γ-tubulin contrasts with results obtained with other anti-centrosomal antibodies which detected their corresponding antigens in the enriched centrosomal fraction only. For example Fig. 1A (left panel) shows the results obtained using the monoclonal antibody CTR210 (Fleury et al., 1993; Perret et al., 1995). We thus conclude from this simple cell fractionation experiment that a significant pool of γ-tubulin is not associated with the centrosome.

The centrosomal γ-tubulin

Biochemical properties of the centrosomal γ-tubulin

Using different chemical treatments which allowed us to establish the relationship between solubilization of centrosomal components and loss of parthenogenetic and in vitro MT-

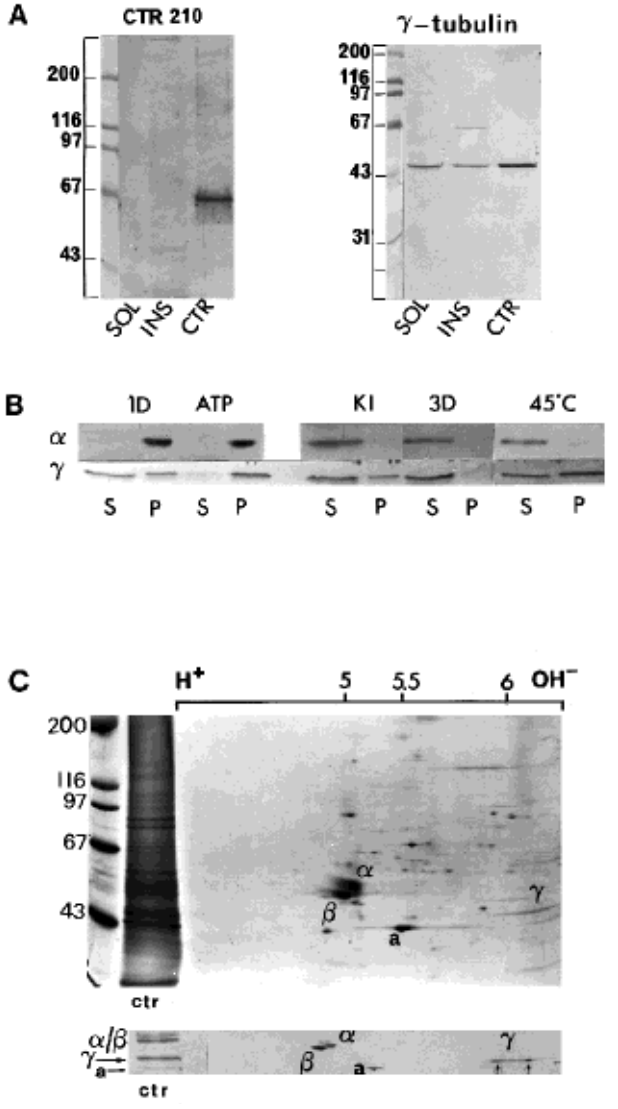

Fig. 1. (A) γ-Tubulin is not an exclusive centrosomal component. Comparison of immunodetection of lymphoblastic cellular proteins with the monoclonal antibody CTR210 (8% SDS-PAGE) and with the affinity purified anti-γ-tubulin IgGs (10% SDS-PAGE), after transfer to nitrocellulose filters. Lane SOL, cellular Triton X-100 soluble proteins (40 μg). Lane INS, cellular Triton X-100 insoluble proteins (40 μg). Lane CTR, isolated centrosomes (4×10^7 centrosomes; 0.6 μg). Note that while the CTR210 antigen is specifically detected in the centrosomal fraction, γ-tubulin, although enriched in centrosome fraction, is also detected in the other fractions. (B) Biochemical extraction of the centrosomal γ-tubulin. Supernatants (S) and pellets (P) obtained after extraction of isolated centrosomes under the conditions indicated on the figure (for details, see Materials and Methods) were separated by SDS-PAGE, transfered to nitrocellulose membrane and immunodetected with either anti-α-tubulin monoclonal antibody or with anti-γ-tubulin IgGs. The position of α-tubulin (α) and of γ-tubulin (γ) are indicated on the left of the figure. The behavior of both α- and γ-tubulins seem to be similar in ATP (no extraction) and 3D (total extraction) conditions, whereas both proteins are differently extracted after 1D, KI and 45°C treatments. (C) 2-D gel analysis of the centrosomal γ-tubulin. Upper panel, silver staining of equilibrium 2-D gel containing centrosomal proteins. Lane ctr, the corresponding one-dimensional protein pattern. The positions of actin (a), α/β- and γ-tubulins are indicated on the gel. Lower panel, sequential immunodetection of actin, α/β-tubulins and then of γ-tubulin. α/β-tubulins and γ-tubulin differ in both molecular mass and pI. Note that γ-tubulin was revealed as two spots (two thin arrows). The pI scale was determined in the immunoblotting experiment. The molecular mass markers (in kDa) are shown on the left of each gel in A and C.

nucleating activities of isolated centrosomes (Klotz et al., 1990; Moudjou et al., 1991), we have attempted to estimate the strength of the γ-tubulin association with centrosomes (Fig. 1B). Treatment of these isolated centrosomes with non ionic detergents failed to further solubilize centrosome-associated γ-tubulin. However, a combination of a non ionic detergent and salt (250 mM NaCl; buffer 1D) solubilized about 60% of the centrosomal γ-tubulin (Fig. 1B, lanes 1D), whereas a treatment with 250 mM NaCl alone had no effect (data not shown). Centriolar α-tubulin was not solubilized under the same conditions (Fig. 1B). The association of γ-tubulin with the centrosome was not affected by a treatment with 10 mM Mg-ATP (Fig. 1B, lanes ATP). The chaotropic agent KI or the 3D buffer, which is more stringent, extracted most of the γ-tubulin. From these results (1D, KI and 3D conditions) we conclude that only a part of γ-tubulin is tightly associated with the centrosome.

Heat treatment of isolated centrosomes, a condition which was shown to extract axonemal MTs from sea urchin (Steffen et al., 1989), largely solubilized α-tubulin (Fig. 1B, lanes 45°C), indicating complete disorganization of centriolar MTs

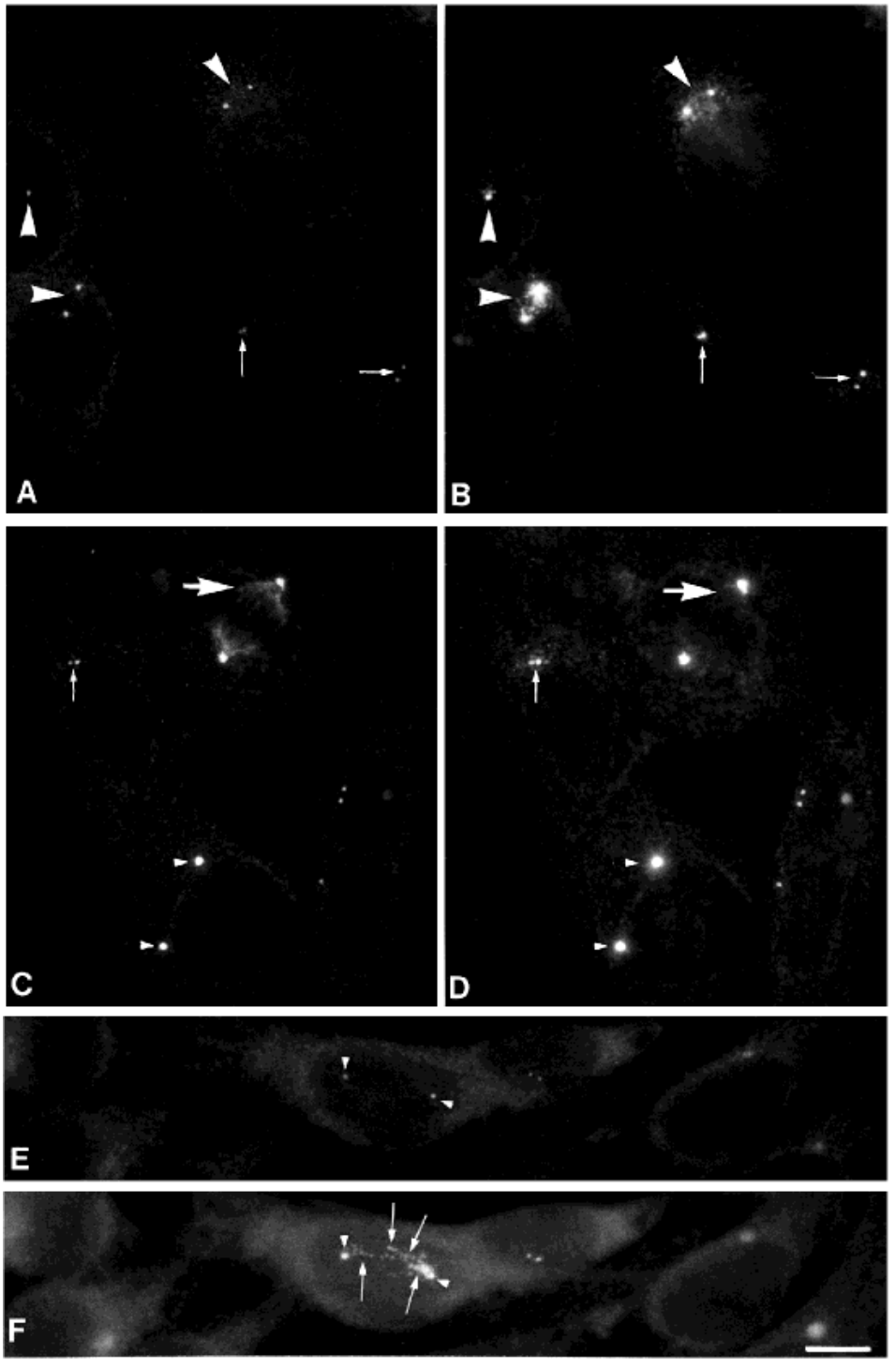

Fig. 2. Immunofluorescence localization of γ-tubulin in HeLa cells. Double immunostaining of HeLa cells using the affinity purified anti-human γ-tubulin IgGs (A,C,E) and the CTR453 anti-PCM mAb (B,D,F). Cells were either pre-extracted with Triton X-100 before methanol fixation (A-D) or directly fixed for 5 minutes with dry methanol at –20°C (E,F). Different stages of the cell division cycle are present in these panels. In all cases, the γ-tubulin staining is less than the CTR453 staining (compare cells indicated with thin arrows in A,B and C,D). Note that in some cells, centrosomes displayed a large distribution of the pericentriolar satellites which were decorated with CTR453 mAb but not with the anti-γ-tubulin antibody (compare cells indicated with arrowheads in A,B), the latter recognized material which seems to be restricted to an area very close to the centrioles. Note also the increase of centrosomal staining with anti-γ-tubulin antibody during the G_2/M transition (arrowheads in C,D). In metaphase cells, γ-tubulin is associated with the mitotic centrosome and also with the proximal MTs of the mitotic spindle (large arrows in C,D). In the same mitotic cell, CTR453 mAb staining is associated with the mitotic spindle poles only. (E and F) A particular cell containing two centrosomes, one of them displays a large distribution of the pericentriolar satellite which is strongly decorated with CTR453 mAb (arrows in F) while in the same cell, the γ-tubulin is much more restricted (arrowheads). Bar, 10 μm.

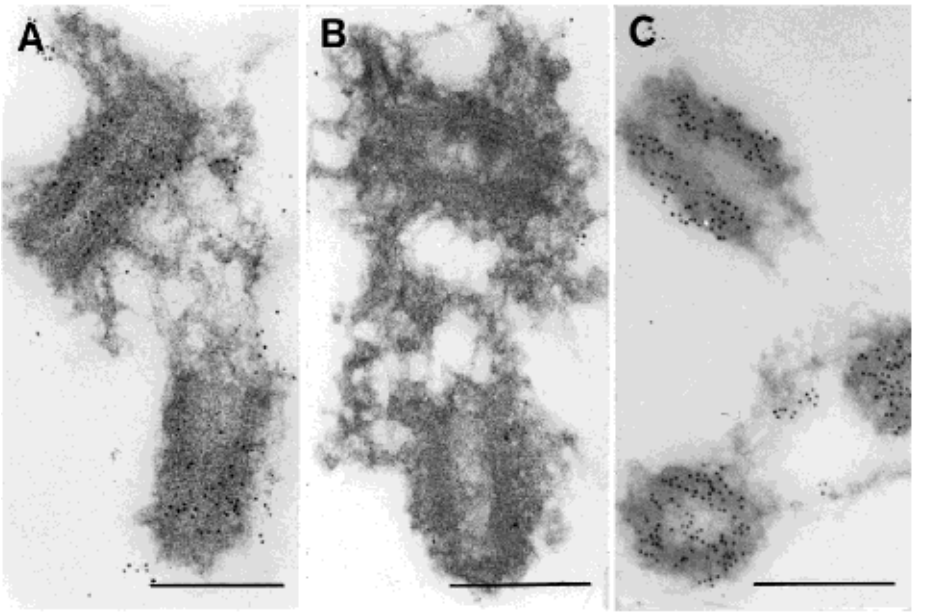

Fig. 3. LR-White post-embedding immunolocalization of γ-tubulin in isolated KE37 human centrosomes. Sections were treated with affinity purified anti-γ-tubulin IgGs (A), with affinity purified anti-γ-tubulin IgGs preincubated with the peptide used as immunogen (B) or with the mAb GT335 (C). The distribution of γ-tubulin is striking: while α/β-tubulins are restricted to centriole wall, γ-tubulin is detected in both the PCM and the centrioles themselves. Preincubation of the anti-γ-tubulin IgGs with the corresponding peptide used as immunogen abolished most of the centrosomal staining (B). No significant staining was detected after incubation of centrosomal sections with the secondary antibodies alone (not shown). Bars, 0.2 μm.

(confirmed by ultrastructural EM observation, not shown). Under the same conditions, only about 30% of γ-tubulin was extracted (Fig. 1B, lanes 45°C). The possibility that this population of γ-tubulin could correspond to a centriole-associated form is discussed below.

We performed 2-D gel electrophoresis of isolated centrosomes in order to establish the IEF pattern of γ-tubulin. Fig. 1C shows silver staining of a 2-D gel of a centrosomal protein sample. Sequential immunodetection with both anti-α and anti-β-tubulin monoclonal antibodies and with anti-γ-tubulin IgGs revealed that γ-tubulin is significantly less acidic (pI around 6) than α/β-tubulins (pI around 5-5.2) (Fig. 1C), a result which corresponds to the calculated pI (5.8) of human γ-tubulin (Stearns et al., 1991). Moreover, we have observed that γ-tubulin signal is elongated and can be resolved as at least two spots after IEF separation (Fig. 1C, two thin arrows).

Ultrastructural immunolocalization of γ-tubulin

Immunolocalization of γ-tubulin by double immunofluorescence approach revealed that the centrosomal staining in HeLa cells was more restricted to centrioles than the staining obtained with the mAb CTR453, a marker of the whole centrosome (Bailly et al., 1989, 1992) (compare Fig. 2A,C,E with B,D,F, respectively). We also observed a specific decoration of the polar spindle MTs (Fig. 2C) as recently reported by Lajoie-Mazenec et al. (1995). To obtain precise information on γ-tubulin localization within the centrosome, we developed a post-embedding approach using isolated centrosomes embedded both in LR-White resin (Fig. 3) or in Unicryl (Fig. 4). As a positive control, we used the monoclonal antibody GT335 directed against the polyglutamylated isoforms of α/β-tubulins (Wolff et al., 1992), which was shown to specifically decorate basal body and axonemal MTs in sperm cells (Fouquet et al., 1994) and centriolar MTs in cultured cells (Bobinnec et al., 1995): gold particles were present along centriolar MT triplets (Fig. 3C). Using the anti-γ-tubulin IgGs, we observed gold particles distributed on the centrosomal fibrillar pericentriolar material, except on the appendages (Fig. 3A). However, gold particles were often concentrated on the centrioles, and were also observed within the lumen of the centrioles. Preincubation of the anti-γ-tubulin IgGs with the peptide used as an immunogen abolished all centrosome specific staining (Fig. 3B). The presence of gold particles close to the centrioles could correspond, however, to a collapsed pericentriolar matrix. As γ-tubulin localization within the centrosome should be an important piece of information for identifying the sites where MTs are nucleated, we attempted in several ways to identify this localization precisely. We first improved the specificity of the immunocytochemical localization by preincubating centrosome sections with an unlabelled goat serum, instead of BSA, before carrying out the double immunogold method. This eliminated any background particles on the centrosome sections. As shown in Fig. 4A, most of the particles were very close to the centriole MTs, to the pro-centriolar MTs, or within the lumen of centrioles. Some were present also in the centrosome matrix away from the centrioles. Using this method, we then looked at γ-tubulin localization in centrosomes isolated from calf thymus, which are known to nucleate MTs from the distal end of both centrioles (Komesli et al., 1989). As shown in Fig. 4B, gold particles were more generally concentrated at the distal end of centrioles, as expected from re-growth experiments. Since part of the centrosomal γ-tubulin is not strongly associated (see above), we are still concerned by the intraluminal localization of gold particles, which could reflect some redistribution during the isolation of centrosomes (see Discussion).

The non centrosomal γ-tubulin

The above biochemical data indicated that most of the γ-tubulin was not associated with the centrosome (Fig. 1A). A crude estimate from western blotting, in which the centrosomal sample represents a 55-fold enrichment compared to soluble protein fractions, would indicate that a minimum figure of 80% of total γ-tubulin is non centrosomal. Indeed, dissociation

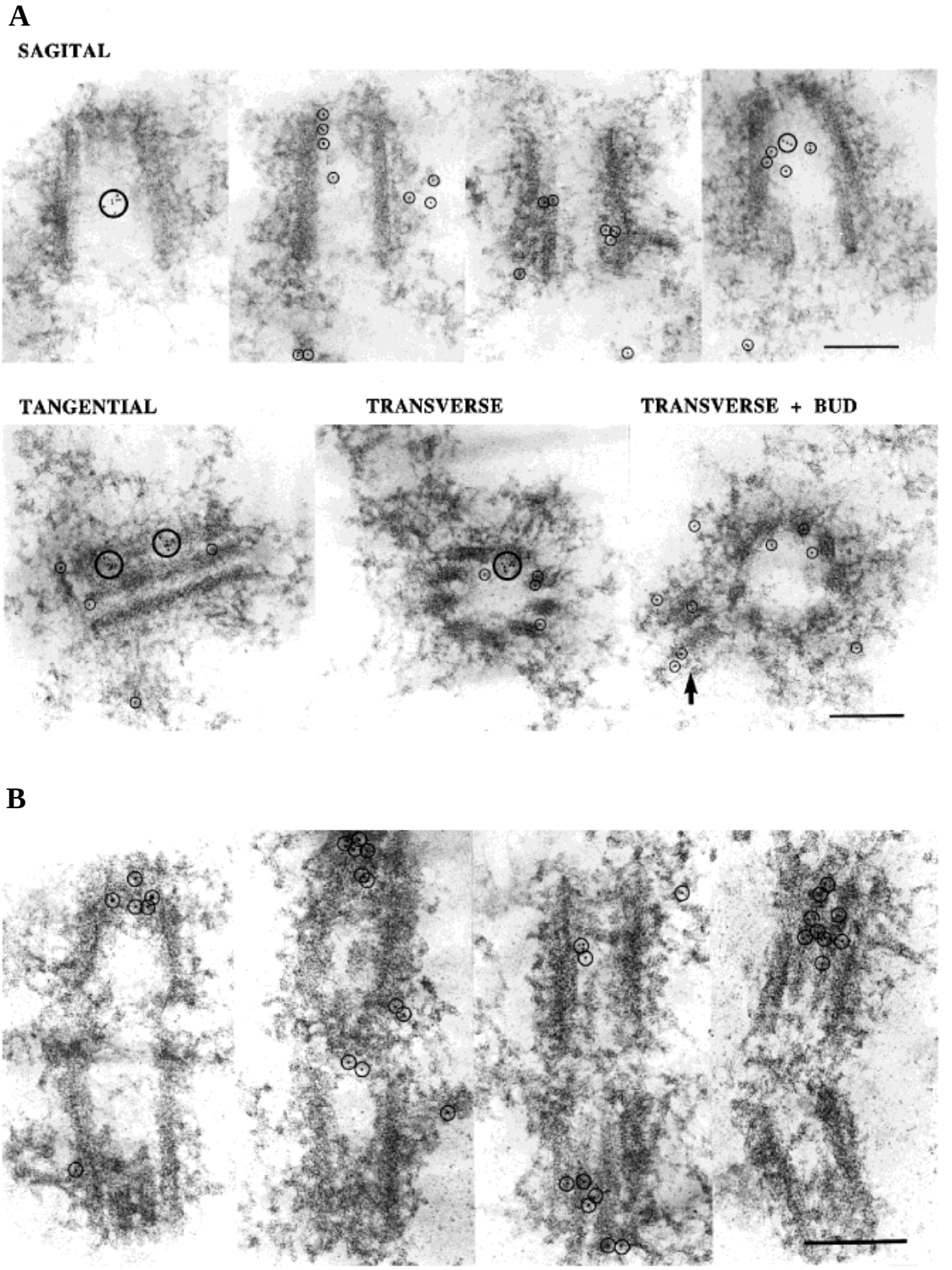

Fig. 4. Unicryl post-embedding immunolocalization of γ-tubulin in centrosomes isolated from (A) human KE37 lymphoblasts or (B) calf thymocytes. Sections were pre-treated with concentrated goat serum (dilution 1/10) before treatment with affinity purified anti-γ-tubulin IgGs. In these conditions, no background particles were observed. Note that most of the gold particles are closely associated with the centriolar MTs and can also be present in the lumen (top left and right in A). When a pro-centriole bud was sectioned, it also demonstrated associated γ-tubulin (arrow in the bottom right of A), suggesting that γ-tubulin could participate in its growth. In B, most of the particles seem concentrated at the distal end of the centrioles where MTs are assembled in that case. Note that Unicryl sections of human centrosomes have been extended, either during heat polymerization of Unicryl or during incubation with the antibodies, whereas those of calf thymus have not. Bars, 0.2 μm.

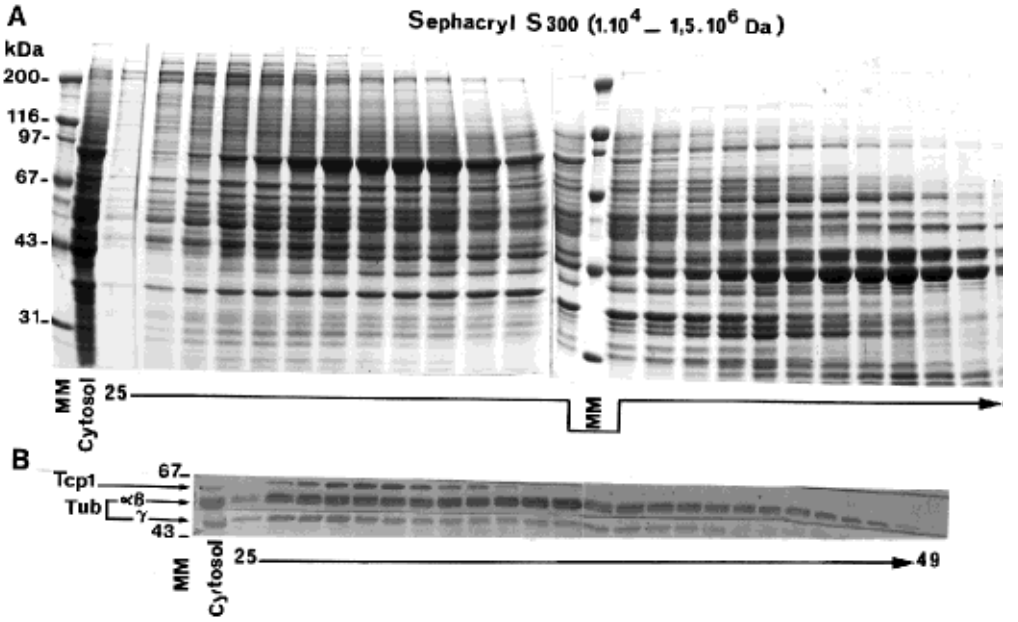

Fig. 5. The cytosolic form of γ-tubulin is involved in high molecular mass complexes. (A) Cytosol obtained from KE37 lymphoblastic cells was loaded on Sephacryl S-300 column and the obtained fractions were assayed for their protein content by Coomassie blue staining of 10% SDS-PAGE. (B) The same fractions as in A were separated using 8% SDS-PAGE, transferred to nitrocellulose filter and the regions containing Tcp1, α/β-tubulins and γ-tubulin proteins were cut up horizontally and processed for western immunoblotting using the corresponding antibodies. Tcp1 protein was eluted as expected, as homogenous large particles of molecular mass 700-900 kDa. γ-Tubulin was detected in fractions 25-41 with an enrichment in the high molecular mass fractions (fractions 25-34). α/β-tubulins were eluted in all fractions.

could occur during isolation, as we have observed that a part of the centrosomal γ-tubulin is weakly associated.

Gel filtration and sucrose gradient centrifugation of cytosolic γ-tubulin

We have shown that γ-tubulin is detected in a Triton X-100 soluble fraction obtained after cell lysis with detergent and low speed centrifugation (see Materials and Methods, Fig. 1A). We further demonstrated that γ-tubulin was also present in a cytosol fraction obtained by mechanical breaking of cells in the absence of detergent and subsequent high speed centrifugation (Fig. 5, lanes cytosol; see also Fig. 8, lane S1). An insoluble form of γ-tubulin (about 50% of total γ-tubulin) was also constantly observed in the high speed sedimentation pellet. We focused our analysis on the cytosolic form of γ-tubulin only. The cytosol fraction was loaded onto a Sephacryl S-300 column, and fractions were collected and analysed by Coomassie blue staining of SDS-PAGE (Fig 5A) and immunodetection using different antibodies (Fig. 5B). Fig. 5B shows that γ-tubulin was present throughout most of the elution profile, the major part eluting at a high molecular mass (fractions 25-32). As it was previously shown that chaperone molecules, especially the chaperonin Tcp1, are involved in the correct folding of cytoskeletal components (Lewis et al., 1992; Yaffe et al., 1992; Gao et al., 1993; Melki et al., 1993; Sternlicht et al., 1993), we looked for the presence of Tcp1 within the γ-tubulin containing high molecular mass fractions. Indeed, Tcp1 was eluted in fractions 27-31 at their expected position of 700-900 kDa. α/β-Tubulins were eluted in all fractions of this column, indicating heterogeneity in the size of the complexes in which they could be involved.

In an attempt to further substantiate the association of γ-tubulin with Tcp1, we pooled fractions 25-32 from the Sephacryl S-300 column and applied them to a 15-40% linear

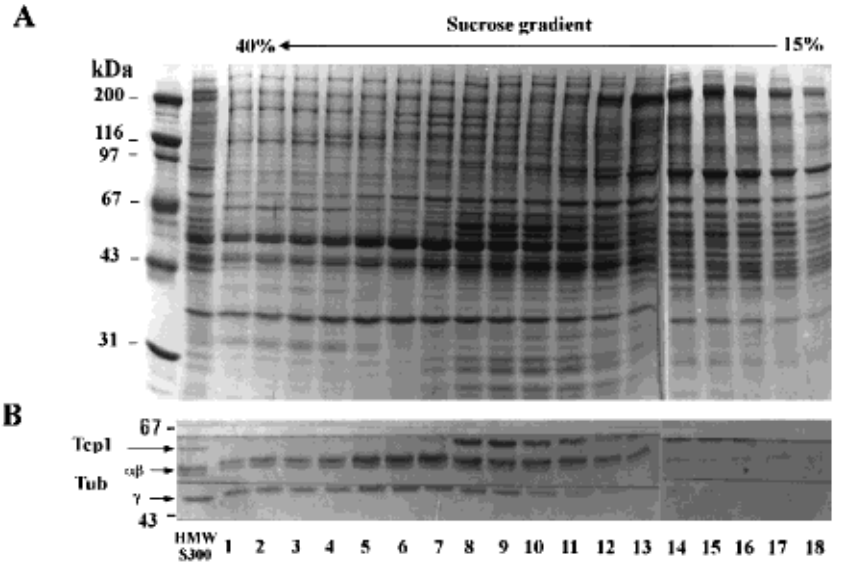

Fig. 6. The cytosolic form of γ-tubulin behaves as heavy complexes on a sucrose gradient. (A) The high molecular mass fractions (lane HMWS300), obtained with the Sephacryl S-300 column shown in Fig. 5, containing the maximum of γ-tubulin, were loaded on a continuous 15-40% sucrose gradient and centrifuged at 100,000 ***g*** for 16 hours. Fractions from the bottom (fraction 1) to the top (fraction 18) of the gradient were recovered and analysed for their protein content by Coomassie blue staining of a 10% SDS-PAGE gel. (B) The same fractions as in A were separated by 8% SDS-PAGE, transferred to a nitrocellulose filter and the regions containing Tcp1, α/β-tubulins and γ-tubulin were cut up horizontally and processed for western immunoblotting using the corresponding antibodies. Note the parallel behaviour of both α/β-tubulins and γ-tubulin which peaked at the heavy fractions (lanes 5-7), higher than the Tcp1 complex which peaked at fraction 8-9.

sucrose gradient that was run for 16 hours at 100,000 ***g***. Fractions were collected from the bottom of the gradient and analysed for their protein composition by SDS-PAGE and by immunoblotting using antibodies directed against γ-tubulin, α/β-tubulin and Tcp1 (Fig. 6). Immunoblotting detection showed that γ-tubulin, as well as α/β tubulins, behaved as high molecular mass complexes peaking at fractions 5 to 7, higher than the Tcp1 complex which peaked at fractions 8-9 (Fig. 6B). A small amount of Tcp1 was also detected in the light fraction (fractions 14-16) possibly corresponding to the monomeric forms of Tcp1 protein. It is noteworthy that fractions 1 to 7 showed a specific enrichment of other polypeptides (Fig. 6A). We conclude that, if any, only a small part of γ-tubulin is associated with Tcp1, whereas the major part is apparently involved in larger cytosolic complexes which might include other proteins and which remain to be characterized.

Immunoprecipitation of cytosolic complexes containing γ-tubulin and TCP1

In an attempt to discriminate a true association of γ-tubulin and Tcp1 from a mere co-sedimentation of these two proteins, we further carried out immunoprecipitation experiments, using two types of starting material, both of them containing the total complement of cytosolic γ-tubulin: either pooled fractions (1 to 12) from a sucrose gradient similar to that shown in Fig. 6; or the protein fraction which bound to a phosphocellulose column when a cytosolic fraction was applied (fraction P11; see Material and Methods). The latter, which contained all the soluble γ-tubulin, all Tcp1, and about 30% of α/β-tubulin, was eluted by one 0.5 M NaCl step (Fig. 7A). As the same immunoprecipitation results were obtained using both types of starting material, only results with the P11 fraction are shown.

Fig. 7 shows an example of immunoprecipitation experiments using anti-α, anti-γ-tubulin and anti-Tcp1 antibodies. Incubation of the P11 fraction with Protein A alone, or with unrelated IgGs, did not precipitate any proteins (Fig. 7A, control lanes). Both anti-α and anti-γ-tubulin antibodies immunoprecipitate γ-tubulin and Tcp1 chaperonin (Fig. 7A-B black arrowhead and short arrow, respectively) in addition to other polypeptides (Fig. 7A). Amongst these other proteins, we identified hsp90 (Fig. 7B, open arrowhead) and hsp70 (Fig. 7B,

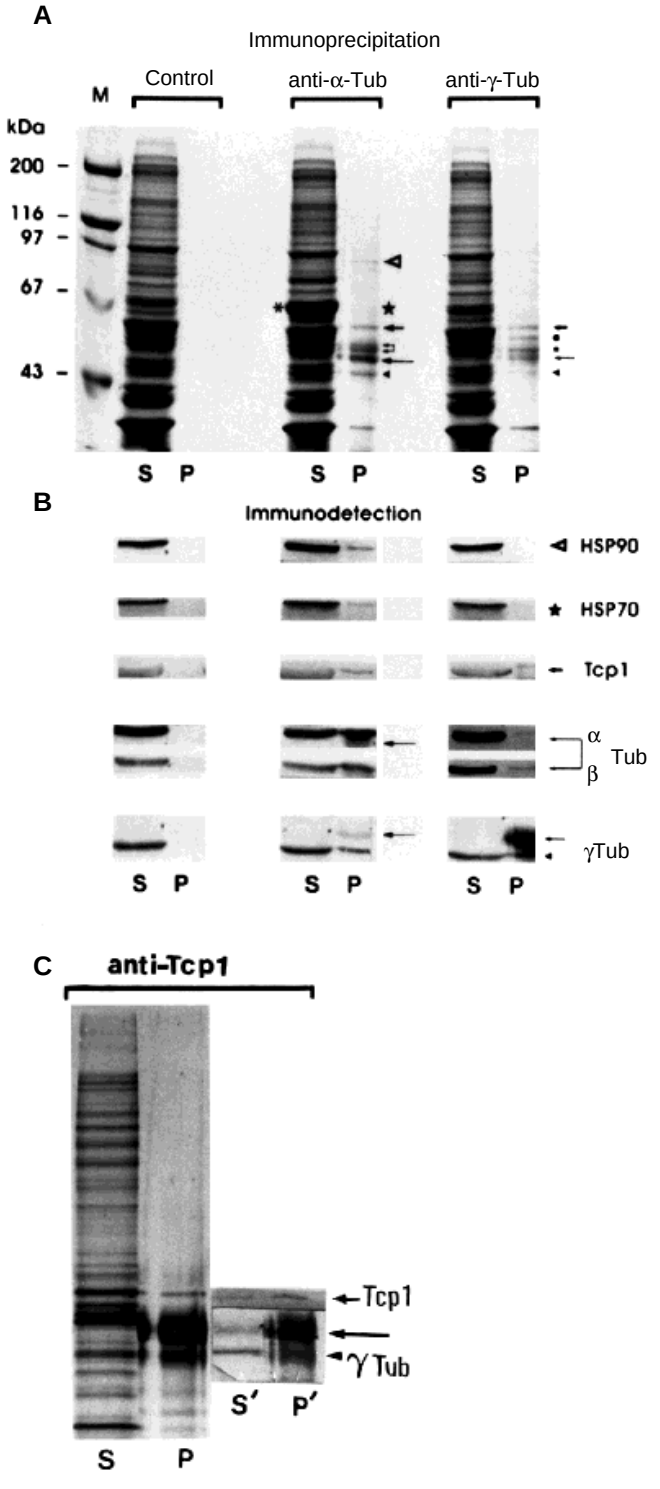

Fig. 7. Immunoprecipitation of the complexes containing α/β- and γ-tubulin. The P11 fraction (see Materials and Methods) was dialysed against 1D buffer and then subjected to immunoprecipitation with anti-α-tubulin, anti-γ-tubulin or anti-Tcp1 antibodies. (A) Coomassie blue staining of 8% SDS-PAGE. Control lanes show supernatant and pellet obtained after incubation of the P11 fraction with Protein A-Sepharose beads alone. Lanes S and P correspond to the supernatants and the immunoprecipitated pellet, respectively, obtained with control, anti-α-tubulin and anti-γ-tubulin. No protein was detected in the control pellet. The asterix shown on the left of lane S of the anti-α-tubulin experiment corresponds to albumin present in the ascite liquid containing anti-α-tubulin IgGs. (B) Same fractions as in A were transferred to a nitrocellulose filter. After Ponceau red staining, the nitrocellulose filter was cut up and each region was then probed with the corresponding antibody to detect the proteins indicated on the right of the panel. The long thin arrows in A and B point to the IgG heavy chains revealed by the anti-rabbit and anti-mouse IgG secondary antibodies depending on the nature of the primary antibody. The symbols used for each protein on the right of B are reported in A to show their position in the protein profile of the immunoprecipitated pellets. The black dot and the black square in A indicate unknown proteins specifically observed in the anti-γ-tubulin immunoprecipitate. (C) Immunoprecipitation with anti-Tcp1 antibody. Lanes S and P correspond to the supernatant and immunoprecipitated pellet, respectively, after staining with silver nitrate. Lanes S′ and P′ correspond to the immunoblot of fractions S and P, respectively, with both anti-Tcp1 (small arrow) and anti-γ-tubulin (arrowhead) antibodies. The large band in lane P′ (long arrow) corresponds to the immunoglobulin heavy chain of the anti-Tcp1 antibody.

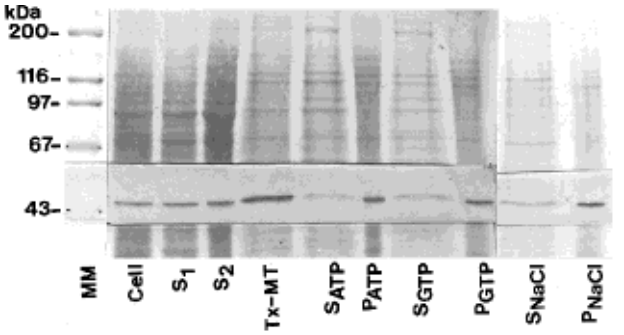

Fig. 8. Association of cytosolic γ-tubulin with taxol-stabilized MTs from KE37 cells. Proteins from different samples obtained during taxol-stabilized MT preparation were separated by 8% SDS-PAGE, transferred to nitrocellulose and stained with Ponceau red. Only the γ-tubulin containing region was cut out and revealed with the anti-γ-tubulin IgGs. Lane Cell, whole cellular proteins. Lane S1, high speed supernatant obtained after lysis of cells. Lane S2, supernatant after addition of taxol, GTP, AMP-PNP and apyrase to S1 and sedimentation of the resulting taxol-stabilized MTs. Lane Tx-MT, taxol-stabilized MTs fraction. Lanes SATP, SGTP and SNaCl show the supernatants obtained after extraction of taxol-stabilized MTs with 10 mM Mg-ATP, 10 mM Mg-GTP or 0.6 M NaCl, respectively. Lanes PATP, PGTP and PNaCl correspond to the resulting pellets, respectively.

star), which are immunoprecipitated with anti-α-tubulin but not with anti-γ-tubulin IgGs. Both antibodies immunoprecipitated a protein of around 37 kDa. Conversely, anti-Tcp1 antibody immunoprecipitated γ-tubulin (Fig. 7C). Interestingly, anti-γ-tubulin IgGs specifically immunoprecipitated two unidentified polypeptides with relative molecular masses of 55 and 58 kDa (Fig. 7A, black dot and square). We have observed that while anti-α-tubulin antibody immunoprecipitated α/β- and γ-tubulin, anti-γ-tubulin IgGs only immunoprecipitated a very small fraction of α/β-tubulin.

We conclude the existence of a true γ-tubulin-Tcp1 complex that, however, represents a minor part of the cytosolic γ-tubulin in human cells.

Binding of cytosolic γ-tubulin to taxol-stabilized MTs

To test whether the cytosolic γ-tubulin from human lymphoblastic cells could interact with MTs, we monitored by immunoblotting the behaviour of γ-tubulin during the preparation of taxol-stabilized MTs. We observed that a fraction of cytosolic γ-tubulin was indeed associated with isolated taxol-stabilized MTs (Fig. 8). This association was not sensitive to extraction with either 10 mM ATP or GTP. It is also resistant to 0.6 M NaCl, suggesting a strong interaction (Fig. 8, lanes NaCl). Since taxol-stabilized MTs were pelleted at 20,000 ***g*** through a 5% sucrose cushion, it is unlikely that these results correspond to a non specific co-sedimentation of cytosolic complexes obtained in a supernatant after centrifugation at 150,000 ***g***.

DISCUSSION

The aim of this work was to clarify the cellular distribution of γ-tubulin in human somatic cells.

γ-tubulin does not behave as a genuine centrosomal component

Our results are in agreement with the idea that a large pool of γ-tubulin is not associated with the centrosome in human lymphoblastic cells, but is involved in large complexes as was shown in *Xenopus* egg and somatic cells and in human 293 cells (Stearns and Kirschner, 1994). Genuine centrosomal antigens are usually undetectable by immunoblotting in whole somatic cell extracts and must be enriched by isolating centrosomes (see Fig. 1A in this work; see also Bouckson-Castaing et al., 1995; Doxsey et al., 1994; Keryer et al., 1993; Moudjou et al., 1991 for other examples). The cytoplasmic pool of γ-tubulin is probably not detected by classical immunofluorescence techniques in somatic cells as the protein might be homogeneously distributed in the cytoplasm. Alternatively, it could be extracted or not accessible to the antibody due to interactions with other proteins. During metaphase, however, γ-tubulin was not restricted to the spindle poles, but could be detected in the polar spindle MTs, as already clearly shown in plant (Liu et al., 1993, 1994) and several animal cells (Lajoie-Mazenec et al., 1994). The broad polar staining observed in HeLa cells could correspond to specific γ-tubulin-containing PCM. However, several of the anti-PCM antibodies did not stain spindle MTs (see Fig. 4D) suggesting that this additional γ-tubulin staining could correspond to cytoplasmic γ-tubulin which specifically associates with the mitotic MTs.

The centrosomal form of γ-tubulin

We have investigated the specific localization and the biochemical properties of centrosomal γ-tubulin. Pre-embedding immuno-gold electron microscopic localization of γ-tubulin in cells, has shown that the main accumulation of centrosomal γ-tubulin was at the PCM (Stearns et al., 1991; Felix et al., 1994). The post-embedding immunolocalization approach used in the present work demonstrated that γ-tubulin is associated with the PCM, but is most often in close association with the centrioles, or with budding centrioles. Moreover, in well oriented sections of centrosomes, we have observed a significant number of gold particles within the lumen of centrioles, an observation which has yet to be convincingly corroborated by in situ postembedding immunolocalization. Double immunofluorescence experiments using anti-PCM and anti-γ-tubulin antibodies also show at the optical level that the γ-tubulin seems to be more closely associated with centrioles than other PCM markers during interphase. The significance of γ-tubulin association with the centrioles themselves can only be speculative with our current state of knowledge of the true function(s) of this protein. A recent report on the three-dimensional characterization of centrosomes from early *Drosophila* embryos has shown that many MTs originate near the centrioles (Moritz et al., 1995). Involvement in centriolar duplication or in the nucleation/stabilisation of the centriolar MTs could be one of its potential roles. Interestingly, recent data suggested that γ-tubulin is required not only for MT nucleation, but also for the structural integrity of the centrosome in *Drosophila* neuroblasts (Sunkel et al., 1995).

The nature of the complex(es) through which γ-tubulin associates with centrosomal proteins is currently being addressed. The presence of molecular chaperones from the hsp70 family (Perret et al., 1995), the hsp90 family and Tcp1 in centrosome preparation (not shown) could be relevant to this question. The

presence of two γ-tubulin isoforms, as judged by 2-D gel analysis, within the isolated centrosomes could be due to post-translational modification such as phosphorylation. Another possibility is that these two isoforms could correspond to the products of two genes. Indeed, two genes have been identified in *Arabidopsis thaliana* (Liu et al., 1994) and in *Drosophila melanogaster* (Wilson et al., 1992; Zheng et al., 1991).

The cytoplasmic forms of γ-tubulin

Using gel filtration and sucrose gradient centrifugation, we observed that the cytosolic pool of γ-tubulin corresponds to fractions of high molecular mass. We also observed that all the soluble form of γ-tubulin bound to phosphocellulose column, and about 30% of cytosolic α- and β-tubulin, suggesting that their retention was due to interactions with other proteins. Both α- and β-tubulin were present in immunoprecipitates obtained with anti-γ-tubulin IgGs but it is difficult to conclude from such a result whether γ-tubulin interacts directly with α- or β-tubulin. Recent results proposed that the basic unit of γ-tubulin is likely to be a monomer or a dimer. The dimeric form could be obtained by treatment of cells extract with a high salt concentration (Stearns and Kirschner, 1994; Vassiliev et al., 1995). Whether this dimeric form corresponds to homo- or heterodimer was not clearly established. We have mainly used a low concentration of salt during protein fractionation in order to avoid dissociation of any salt-dependent γ-tubulin-containing complexes (see also Raff et al., 1993). We have also observed that centrifugation of the cytosolic fraction in sucrose gradients containing a high salt concentration results in dissociation of the heaviest γ-tubulin fractions, while the γ-tubulin comigrating with Tcp1 (see Fig. 6) was not affected (data not shown). The same results were also obtained for α- and β-tubulin-containing high molecular mass fractions.

All together, our results suggest that γ-tubulin is probably involved in different complexes. One of them, identified in the present work, is likely to correspond to a binary combination of γ-tubulin and Tcp1: we have observed a consistant comigration of part of γ-tubulin with Tcp1 after gel filtration chromatography and a cosedimentation in sucrose gradient; immunoprecipitation experiments with anti-γ-tubulin IgGs immunoprecipitated Tcp1; and, reciprocally, anti-Tcp1 antibody immunoprecipitated γ-tubulin. It is known indeed that Tcp1 can form a binary complex with a substrate (Gao et al., 1992, 1993). It is interesting to note that disruption of the yeast Tcp1 gene results in accumulation of multinucleate and anucleate cells which contain morphologically abnormal MT arrays (Ursic and Culbertson, 1991).

γ-Tubulin-containing fractions heavier than the TCP1 particle have been observed in this work, suggesting the existence of other complexes of higher molecular mass. We have demonstrated that anti-γ-tubulin IgGs specifically immunoprecipitated two unidentified polypeptides with relative molecular masses of 55 and 58 kDa. Interestingly, Zheng et al. (1995) have quite recently reported the characterization of a γ-tubulin-containing ring complex, the so-called γ-TuRC, from a mitotic *Xenopus* egg extract, which contains at least seven unidentified polypeptides. This complex has an open ring structure that could provide a minus end template for microtubule assembly. No other complexes, such as Tcp1-γ-tubulin-containing particles were identified in this study nor in the report by Stearns and Kirschner (1994) on the same experimental system, a situation which seems different (see also Vassiliev et al., 1995) from what we observed in somatic cells.

We also observed that anti-α-tubulin antibody efficiently immunoprecipitated α-, β- and γ-tubulin together with hsp70 and hsp90 proteins. Although we have not yet analyzed the significance of these results, they are reminiscent of those reported by Marchesi and Ngo (1993), who have described an in vitro assembly assay of two sets of multiprotein complexes from cytosol of mitotic arrested CHO cells. These complexes are heterogenous in size and contain α-, β- and γ-tubulins, a 50 kDa protein identified as elongation factor EF1a, other minor unidentified polypeptides and either a cognate form of hsp70 or actin, depending on the presence or absence of ATP, respectively. Interestingly, a complex containing centrin, another centrosomal protein, and hsp70 and hsp90 has been recently identified in CSF-arrested *Xenopus* oocytes. This complex, which dissociates following oocyte activation, could be a precursor form in the centrosome assembly pathway (Uzawa et al., 1995).

Our data have revealed unknown proteins immunoprecipitated with anti-α- and anti-γ-tubulin antibodies (see Fig. 7A). Their characterization will be for future work. It will be interesting to determine whether any of these correspond to other centrosomal proteins. Indeed, Raff et al. (1993) have reported that a population of the *Drosophila* embryo γ-tubulin is a part of a complex containing two previously identified *Drosophila* centrosomal/nuclear components named DMAP60 and DMAP190.

The significance of cytoplasmic γ-tubulin

The γ-tubulin-Tcp1 complex identified in the present work could correspond to the structure responsible for the folding of the newly synthesised γ-tubulin observed in vitro by Melki et al. (1993). Once folded from this complex, γ-tubulin could be directed to the centrosome and thus ensure γ-tubulin turnover. The mechanism by which centrosomal proteins are incorporated into the centrosome still remains to be determined. Intermediate complexes in the pathway of targeting centrosomal components to their final localization could indeed exist. Some intermediates could contain other chaperone proteins such as hsp70 (Marchesi and Ngo, 1993). Alternatively, these cytosolic complexes may play a role in sequestering centrosomal proteins until the assembly of a new centrosome is initiated (Uzawa et al., 1995). Interestingly, a protein which belongs to the hsp70 family has also been localized at the centrosome in the dinoflagellate *C. cohnii* and possibly in different cultured animal cells (Perret et al., 1995). The possibility that a specific activity is present within cytosolic γ-tubulin-containing complexes, such as nucleation/stabilization of MTs cannot be eliminated at this stage. Indeed, we have shown that heavy γ-tubulin forms display a high affinity for taxol-stabilized MTs (see also Raff et al., 1993; Stearns and Kirschner, 1994).

In conclusion, the cellular distribution of γ-tubulin in human somatic cells does not correspond to that of a genuine centrosomal component as it was previously thought. It is clear now that the major part of this protein is present in the cytosol, apparently as very large complexes, one of them corresponding to the Tcp1-γ-tubulin particle. Biochemical and structural characterization of the other γ-tubulin-containing complexes, in particular the search for other centrosomal components, determination of the associated activities, and the study of how

their assembly is regulated during the cell cycle, are all questions of great interest. The centrosomal form of γ-tubulin, and its unexpected concentration on centrioles, will also deserve further attention. Answers to all these questions would considerably advance understanding on how γ-tubulin could operate in the cell.

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