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Source: Zoological Science, 15(4) : 477-487

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/0289-0003(1998)15[477:IOAOTW]2.0.CO;2

ZOOLOGICAL SCIENCE **15**: 477–487 (1998)

Interaction of an Overexpressed γ-Tubulin with Microtubules *In Vivo* and *In Vitro*

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ABSTRACT—γ-Tubulin is an ubiquitous MTOC (microtubule-organizing center) component essential for the regulation of microtubule functions. A 1.8 kb cDNA coding for γ-tubulin was isolated from CHO cells. Analysis of nucleotide sequence predicts a protein of 451 amino acids, which is over 97% identical to human and *Xenopus* γ-tubulin. When CHO cells were transiently transfected with the γ-tubulin clone, epitope-tagged full-length, as well as truncated polypeptides (amino acids 1-398 and 1-340), resulted in the formation of cytoplasmic foci of various sizes. Although one of the foci was identified as the centrosome, the rest of the dots were not associated with any other centrosomal components tested so far. The pattern of microtubule organization was not affected by induction of such γ-tubulin-containing dots in transfected cells. In addition, the cytoplasmic foci were unable to serve as the site for microtubule regrowth in nocodazole-treated cells upon removal of the drug, suggesting that γ-tubulin-containing foci were not involved in the activity for microtubule formation and organization. Using the monomeric form of *Chlamydomonas* γ-tubulin purified from insect Sf9 cells (Vassilev *et al*., J. Cell Sci. 108: 1083, 1995), interaction between γ-tubulin and microtubules was further investigated by immunoelectron microscopy. Microtubules incubated with γ-tubulin monomers *in vitro* were associated with more gold particles conjugated with γ-tubulin than in controls where no exogenous γ-tubulin was added. However, binding of γ-tubulin to microtubules was not extensive and was easily lost during sample preparation. Although γ-tubulin was detected at the minus end of microtubules several times more frequently than the plus end, the majority of gold particles were seen along the microtubule length. These results contradict the previous reports (Li and Joshi, J. Cell Biol. 131: 207, 1995; Shu and Joshi, J. Cell Biol. 130: 1137, 1995), which might be ascribed to the difference in the level of protein expression in transfected cells.

INTRODUCTION

The temporal and spatial distribution of microtubules is controlled by structures called microtubule-organizing centers (MTOCs), or centrosomes, which are composed of a pair of centrioles and a surrounding amorphous cloud of pericentriolar material. The macromolecular composition of the centrosome has been analyzed with the aim to understand the mechanism and regulation of microtubule functions (Kimble and Kuriyama, 1992 for a review). The most widely believed and most extensively analyzed MTOC-component is γ-tubulin which was originally identified by genetic screening of suppressors of an *Aspergillus* β-tubulin mutation (Oakley *et al*., 1990). Sequence analysis showed that γ-tubulin is nearly as similar to α- and β-tubulins as they are to each other, indicating that γ-tubulin is a new member of the tubulin superfamily (Burns, 1995a for a review). The importance of the γ-tubulin protein in the regulation of microtubule function has been documented by analysis of mutations of the γ-tubulin gene in fungi (Oakley *et al*., 1990; Stearns *et al*., 1991; Horio *et al*., 1991) and *Drosophila* (Sunkel *et al*., 1995), as well as by suppression of the protein's functions by antibody injection into living cells (Joshi *et al*., 1992). γ-Tubulin is a well-conserved molecule, thus it is functionally exchangeable among species (Horio and Oakley, 1994). However, the sequences related to the γ-tubulin protein in *Saccharomyces cerevisiae* (Tub4p) and *C. elegans* are more divergent than other γ-tubulins (Burns, 1995b for a review). In fact, elimination of the Tub4 function was not rescued by γ-tubulin derived from human, *Xenopus* and *S. pombe* (Spang *et al*., 1996; Marschall *et al*., 1996).

In an effort to understand the role of γ-tubulin in the structure and function of the centrosome, we have cloned cDNA encoding γ-tubulin and expressed the protein in insect Sf9 cells using the baculovirus expression system (Vassilev *et al*., 1995). It was shown that newly synthesized γ-tubulin molecules

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are fractionated into a TCP1 chaperonin complex which released folded γ-tubulin polypeptide in a monomeric form. Such released γ-tubulin monomers were capable of binding to microtubules *in vitro* and biochemical quantities of active monomers were purified using a combination of size-exclusion and ion-exchange column chromatography (Vassilev *et al.*, 1995). Unlike expressed γ-tubulin in a monomeric form, however, endogenous γ-tubulin was shown to exist as a dimer, suggesting that γ-tubulin could form either a homodimer with another monomer of γ-tubulin, or a heterodimer with different kinds of γ-tubulin-like protein or hitherto unknown molecule(s).

To gain more insight into the mechanism and regulation of γ-tubulin function, we have cloned cDNA coding for γ-tubulin in CHO (Chinese hamster ovary) cells and expressed proteins in mammalian cells by transient transfection. Here, we report that overinduced full-length, as well as truncated polypeptides resulted in the formation of cytoplasmic foci in transfected CHO cells. Those γ-tubulin-containing dots did not apparently participate in microtubule assembly *in vivo*, and normal arrays of the microtubule network were retained in the transfected cells. Immunoelectron microscopic analysis further suggested that interaction of *Chlamydomonas* γ-tubulin monomers with microtubules was not extensive and was easily lost during sample preparation. In addition, the majority of immunogold particles were found along the length of microtubules. The results appear to disagree with the previous report (Li and Joshi, 1995) in which γ-tubulin synthesized in reticulocyte lysates was shown to be capable of specific binding to the minus-end of microtubules in an *in vitro* system. The difference in implication of the result in the two systems are discussed.

MATERIALS AND METHODS

Cloning of cDNA coding for γ-tubulin from CHO cells

cDNAs coding for the full-length of γ-tubulin in CHO cells (submitted to EMBL) were isolated in three steps (Fig. 1A). mRNA was first prepared from CHO cells, then cDNA synthesis was initiated with the degenerate antisense oligo primer #1 (P1: VLDVMRRE, amino acids 290-297) corresponding to a conserved domain from known γ-tubulin sequences. Purified cDNA was used as a template for PCR using primers #1 and #2 (P2: DRKDVFFYQ, amino acids 46-54). A 0.7 kb PCR product (clone: CHO-γ-1/2) was re-amplified with nested primers #3 (P3: YPGYMNND, amino acids 245-252) and #4 (P4: LYNPENIY, amino acids 85-92) to confirm the authenticity of the γ-tubulin sequence. The CHO-γ-1/2 clone was next radiolabeled with ^{32}P to screen a CHO cell cDNA expression library cloned in λUni-Zap (Stratagene, La Jolla, CA, USA) which yielded one positive clone with a 1.2 kb insert (clone: CHO-γ-UZ). The remaining 5' end of the gene (clone: CHO-γ-5') was isolated using the 5' RACE method (Kuriyama *et al.*, 1995) with antisense primers #5 (P5: nucleotides 299-322) and #6 (P6: nucleotides 260-280). Double-stranded DNA was sequenced by the dideoxy chain termination method (Sanger *et al.*, 1977), using the Sequenase kit (US Biochemical Co., Cleveland, OH, USA).

Immunological analysis of γ-tubulin expressed in transfected CHO cells

The cDNA fragments encoding the full-length, Δ1 (*Hin*d III fragment: amino acids 1-398) and Δ2 (*Bam*H I fragment: amino acids 1-340), of γ-tubulin were isolated and subcloned into the multicloning site of the cytomegalovirus-based vector, pCMV-4. The plasmid was engineered to include the nucleotide sequence corresponding to the hemagglutinin (HA) epitope so that expressed proteins were attached to HA at the N-terminal end.

Transfection of CHO cells was performed by employing three protocols: microinjection, electroporation and lipofection. All procedures provided essentially identical results. DNA injection into CHO cells grown on photoetched glass coverslips (Bellco, Vineland, NJ, USA) was done as described by Compton and Cleveland (1993). Plasmid DNA 100-500 μg/ml was prepared in the injection buffer (100 mM KCl, 10 mM KPO_4 at pH 7.4) and introduced directly into interphase nuclei using a Narishige micromanipulator. For electroporation, trypsinized cells were placed in a 0.2 mm gap cuvette at 3-8 $\times$ 10^6 cells/ml, then mixed with plasmid DNA at a final concentration of 50 μg/ml. A pulse of 700 μF at 300 V was applied to cells by an Electro Cell Manipulator 600 (BTX, San Diego, CA, USA) (Ausubel *et al.*, 1993). Lipid-mediated DNA transfection (Felgner *et al.*, 1987) was performed using 0.6-1.0 μg/ml plasmid DNA mixed with Lipofectamine Reagent (Life Technologies, Gaithersburg, MD) according to the manufacture's protocol. After 5 hr incubation at 37°C, the medium was removed and cells were cultured for an additional 12-24 hr before fixation.

Protein induction in transfected cells was analyzed by both immunofluorescence staining and immunoblot analysis as described previously (Kuriyama *et al.*, 1995). Briefly, transfected cells plated on coverslips were fixed with methanol at –20°C for 5 min, then rehydrated with 0.05% Tween-20-containing phosphate-buffered saline (PBS-Tw20). After double immunostaining with monoclonal anti-HA (12CA5; Berkeley Antibody Co., Richmond, CA, USA) and affinity-purified rabbit polyclonal anti-γ-tubulin (Vassilev *et al.*, 1995) antibodies, cells were incubated with a mixture of fluorescein-conjugated anti-mouse and Texas Red-conjugated anti-rabbit secondary antibodies. Additional antibodies used to probe for microtubules and centrosomal proteins included monoclonal anti-chicken β-tubulin antibodies (Amersham, Arlington Height, IL, USA), human autoantibody, pericentrin (Doxsey *et al.*, 1994), mouse monoclonal anti-CHO1/MKLP1, CHO2 (Sellitto *et al.*, 1992) and rabbit polyclonal anti-Cep135 (Ohta *et al.*, manuscript in preparation) antibodies.

Immunoelectron microscopy

Protein preparations: The monomeric form of γ-tubulin was purified from Sf9 cells infected with baculovirus containing the *Chlamydomonas* γ-tubulin sequence as described previously (Vassilev *et al.*, 1995). The final buffer composition was 100 mM Pipes at pH 6.8, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM GTP (microtubule buffer) containing 1 mM leupeptin, 1 mM PMSF and 10% glycerol. Flagellar axonemes were prepared from *Chlamydomonas* (Porter *et al.*, 1992), and tubulin subunits capable of polymerization into microtubules *in vitro* were fractionated by sonication of purified outer doublet microtubules (Kuriyama and Linck, 1995). Microtubules were reconstructed in the presence of 10 μg/ml taxol at 37°C.

Binding assays by immunoelectron microscopy: Binding of polyclonal γ-tubulin and monoclonal α-tubulin (Amersham, Arlington Height, IL, USA) antibodies with microtubules was done both in solution and on grids. A solution mixture of γ-tubulin and microtubules at a final concentration of 10-50 μg/ml and 0.4-1.0 mg/ml, respectively, was incubated at 37°C for 15-30 min, then fixed with 0.75% glutaraldehyde in the microtubule buffer. After mounting on nickel grids coated with Pioloform (Ted Pella, Inc., Redding, CA, USA) and carbon, the sample was processed for immunogold staining according to the procedure of Bernstein *et al.* (1994). For assays on grids, microtubules mounted on grids were incubated with the γ-tubulin-containing solution at 37°C for 15-30 min, and were further stained with γ-tubulin antibodies tagged with gold particles as above. After fixation with glutaraldehyde and stained with 1% uranyl acetate, the sample was directly observed by JEOL 100CX electron microscopy. The number of

gold particles were counted and the length of microtubules measured manually in micrographs. The distance between particles in microtubules was calculated as total length of microtubules divided by the total number of associated gold particles. Standard statistical tests were applied to these measurements including t-tests for independent and dependent series. Two to six experiments were done for each assay.

RESULTS

Cloning and sequence analysis of γ-tubulin in CHO cells

Using the cloning strategy outlined in Materials and Methods (Fig. 1A), we isolated cDNA clones coding for the full length of γ-tubulin in CHO cells. The longest open reading frame predicts a protein of 451 amino acids with a calculated molecular mass of 50 kDa (Fig. 1B). The predicted amino acid sequence of the CHO γ-tubulin is 98 and 97% identical to γ-tubulin in human and *Xenopus*, respectively. High homology between these species is seen even at the C terminus, a region known to be quite variable among species (Burns, 1995a). A lesser degree of identity is detected compared to *Drosophila* (79%), fission yeast (77%) and *Aspergillus* (67%). It is reported that *Saccharomyces cerevisiae* and *C. elegans* contain γ-tubulin-like molecules whose primary sequence is more divergent than other γ-tubulin; identity of CHO γ-tubulin to these species is in the range of 41-42%.

Comparisons were also made with α- (α1: Elliott *et al*., 1986), β- (β1: Boggs and Cabral, 1987) subunits of tubulin in the same species. γ-Tubulin is 27 and 31% identical to α- and β- tubulins, respectively, while α and β posses 39% identity in CHO cells. These results is consistent with the previous notion that γ-tubulin is as similar to α- and β-subunits as α- and β-subunits are to each other.

Induction of the full-coding and truncated polypeptides of CHO γ-tubulin in CHO cells by transient transfection

By constructing mammalian expression vectors, we expressed HA epitope-tagged γ-tubulin polypeptides in CHO cells by transient transfection. After confirmation of the 50 kDa protein expression by HA antibody on immunoblots (data not shown), intracellular distribution of exogenous γ-tubulin was analyzed by immunofluorescence microscopy. Figure 2 includes cells fixed after overnight protein induction: expression of γ-tubulin causes cells to induce cytoplasmic foci with variable sizes (A', A" and B'). Although the number of such γ-tubulin-containing dots was variable among cells, an average 4-10 foci were typically seen per cell. The longer the induction, the greater the number and the larger the size of dots induced. Some dots were located at the juxtanuclear position, and they were always positive in immunostaining with other centrosomal antibodies (arrows in Fig. 4), indicating that a part of expressed epitope-tagged γ-tubulin was recruited to the centrosome. It is noteworthy that despite the presence of prominent γ-tubulin-containing structures in the cytoplasm, the transfected cells exhibited normal arrays of microtubules indistinguishable from those in control cells (Fig. 2B").

To identify the domain necessary to induce dots in transfected cells, we prepared two kinds of deletion constructs: Δ1 (*Hin*d III fragment) encodes γ-tubulin with the length of 398 amino acid residues at the N terminus, and the N-terminal 340 amino acids are encoded by the Δ2 (*Bam*HI fragment) clone (Fig. 1A). Transfected cells expressed 44 and 37 kDa polypeptides which were labeled by both HA and γ-tubulin antibodies by immunoblot analysis (data not shown). Like the full-coding sequence of γ-tubulin, both types of truncated γ-tubulin polypeptides were able to induce cytoplasmic foci (Fig. 3), and their expression did not cause significant alteration in microtubule organization in transfected cells. Thus, the sequence of the N-terminal three fourths of the γ-tubulin protein seems enough to induce additional γ-tubulin-containing dots in the transfected cell cytoplasm.

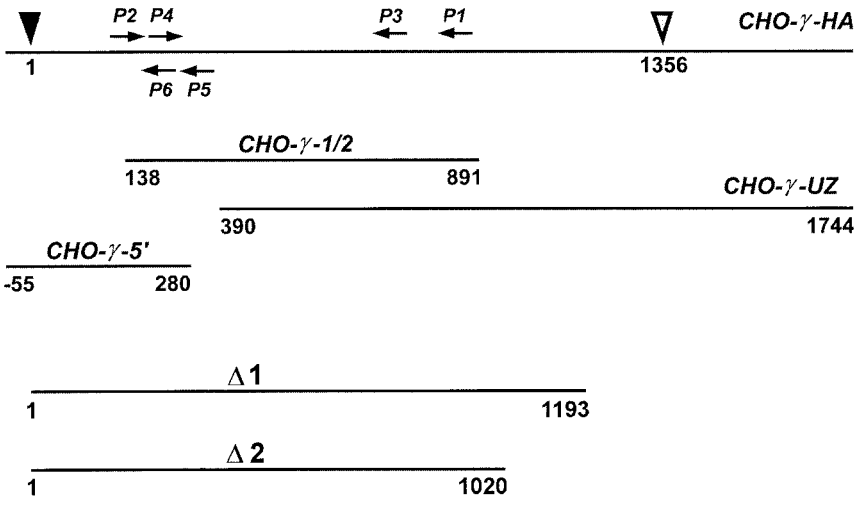
γ-Tubulin-containing cytoplasmic foci are not associated with other centrosomal antigens and microtubule-nucleating capacities

To address the question of how the foci induced by overexpression of γ-tubulin relate to the intrinsic centrosome, we carried out following experiments.

I) Immunostaining of γ-tubulin-containing dots with centrosomal antibodies: Figure 4 illustrates double immunostaining of transfected cells expressing the full-length of γ-tubulin with anti-γ-tubulin antibody (A' to D') and centrosomal antibodies, including pericentrin (A"), Cep135 (B"), CHO1/MKLP1 (C"), and CHO2 (D"). Pericentrin and Cep135 are attached to the centrosome tightly (Doxsey *et al*., 1994; Ohta *et al*., unpublished). In contrast, CHO1/MKLP1 and CHO2 are kinesin-like microtubule motors (Nislow *et al*., 1992; Kuriyama *et al*., 1995) which become associated with the interphase centrosome in a microtubule-dependent manner. The centrosome located next to each nucleus was clearly seen by the antibodies (A"-D"). Although all the antibodies revealed the presence of the centrosome next to each nucleus (arrows in A'-D'), they failed to stain additional γ-tubulin-containing cytoplasmic dots induced in the transfected cells. It is, therefore, concluded that γ-tubulin-containing cytoplasmic foci are not associated with other centrosomal antigens.

II) Reassembly of microtubules onto the cytoplasmic dots: Transfected cells were pretreated with 1.0-2.5 μg/ml nocodazole for 2-5 hr to completely depolymerize *in situ* microtubules. Microtubules started to nucleate around the centrosome upon removal of the drug (Fig. 5). Short microtubules were assembled onto the centrosome within a couple of min after washing out nocodazole from the medium (A"-C"); one, sometimes two dots in the juxtanuclear position were always seen to be associated with repolymerized microtubule asters. These γ-tubulin-containing dots were also positive in immunostaining with other centrosomal antibodies analyzed above. A majority of cytoplasmic foci were, however, incapable of initiation of microtubule assembly. Moreover, the formation of cytoplasmic dots did not cause any changes in the pattern of microtubule organization in the transfected cell. Taken together, these results suggest that extra γ-tubulin foci induced in the cytoplasm are not directly involved in microtu-

A



B

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-55                                                                  1
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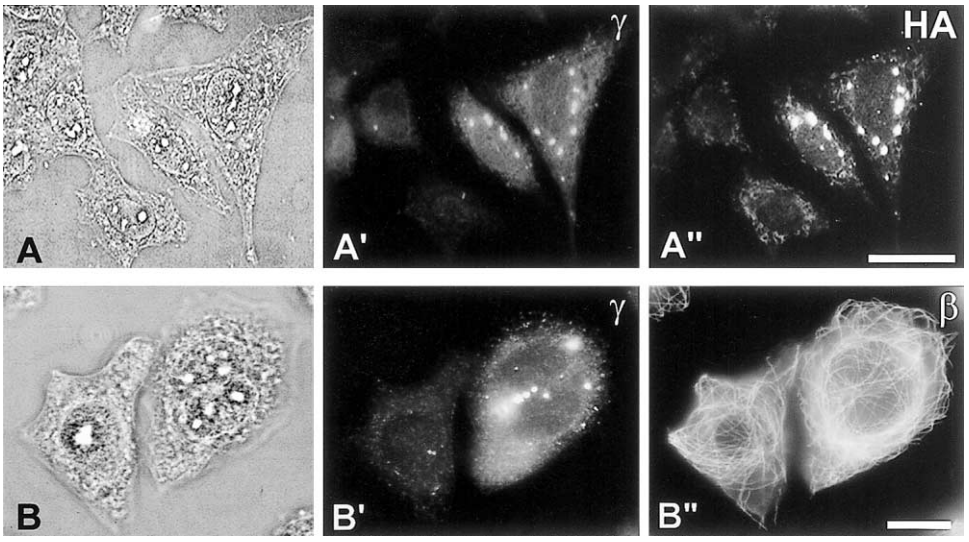

Fig. 2. Immunofluorescence staining of transiently transfected CHO cells expressing the epitope-tagged, full-coding CHO γ-tubulin sequence. Cells were double staining with polyclonal anti-γ-tubulin (**A'**, **B'**) and monoclonal anti-HA (**A"**) or anti-β-tubulin (**B"**) antibodies. Transfected cells with a number of cytoplasmic foci in various sizes contain normal arrays of the microtubule network. Bars, 10 μm (**A**-**A"**), 5 μm (**B**-**B"**).

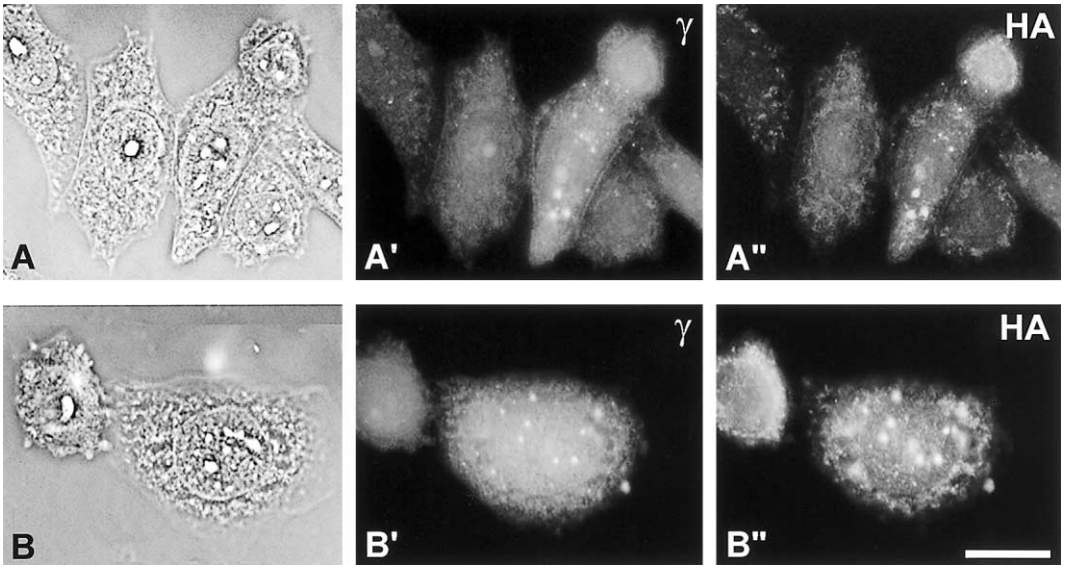

Fig. 3. Immunofluorescence staining of CHO cells expressing truncated polypeptides of γ-tubulin encoded by Δ1 (amino acids 1-398) (**A**-**A"**) and Δ2 (amino acids 1-340) (**B**-**B"**) clones. The same cells were seen by phase-contrast (**A**, **B**) and fluorescence microscopy after double staining with γ-tubulin (**A'**, **B'**) and HA (**A"**, **B"**) antibodies. Like in cells expressing the full length γ-tubulin, cells with excess amounts of N-terminal 88% and 76% coding sequence of the CHO γ-tubulin protein form foci in the cytoplasm. Bar, 10 μm.

Fig. 1. Schematic diagram of cDNA clones and the primary sequence of γ-tubulin in CHO cells. (**A**) Map of cDNAs coding for CHO γ-tubulin. The filled and open arrowheads indicate the position of the start and stop codons, respectively. Δ1 and Δ2 encode truncated polypeptides of γ-tubulin expressed in CHO cells by transient transfection. The numbers underneath at the end of each clone represent nucleotide positions encoded by the clones. Arrows correspond to the nucleotide primers (P1 to P6) used for PCR and RT-PCR for cloning of γ-tubulin cDNAs. (**B**) Complete DNA sequence of γ-tubulin in CHO cells. The deduced amino acid sequence indicated by the single letter code below the DNA sequences.

bule assembly.

In vitro interaction of microtubules with purified γ-tubulin monomers

Figure 6 includes *Chlamydomonas* flagellar axonemes (A) and reconstituted calf brain microtubules (B) incubated with anti-α-tubulin antibody-conjugated with gold particles. Both types of microtubules are covered with gold particles with little background on the supporting film. 250-600 and 40-60 particles were detected per μm of axonemes and brain microtubules, respectively. Since approximately 2,000 tubu-

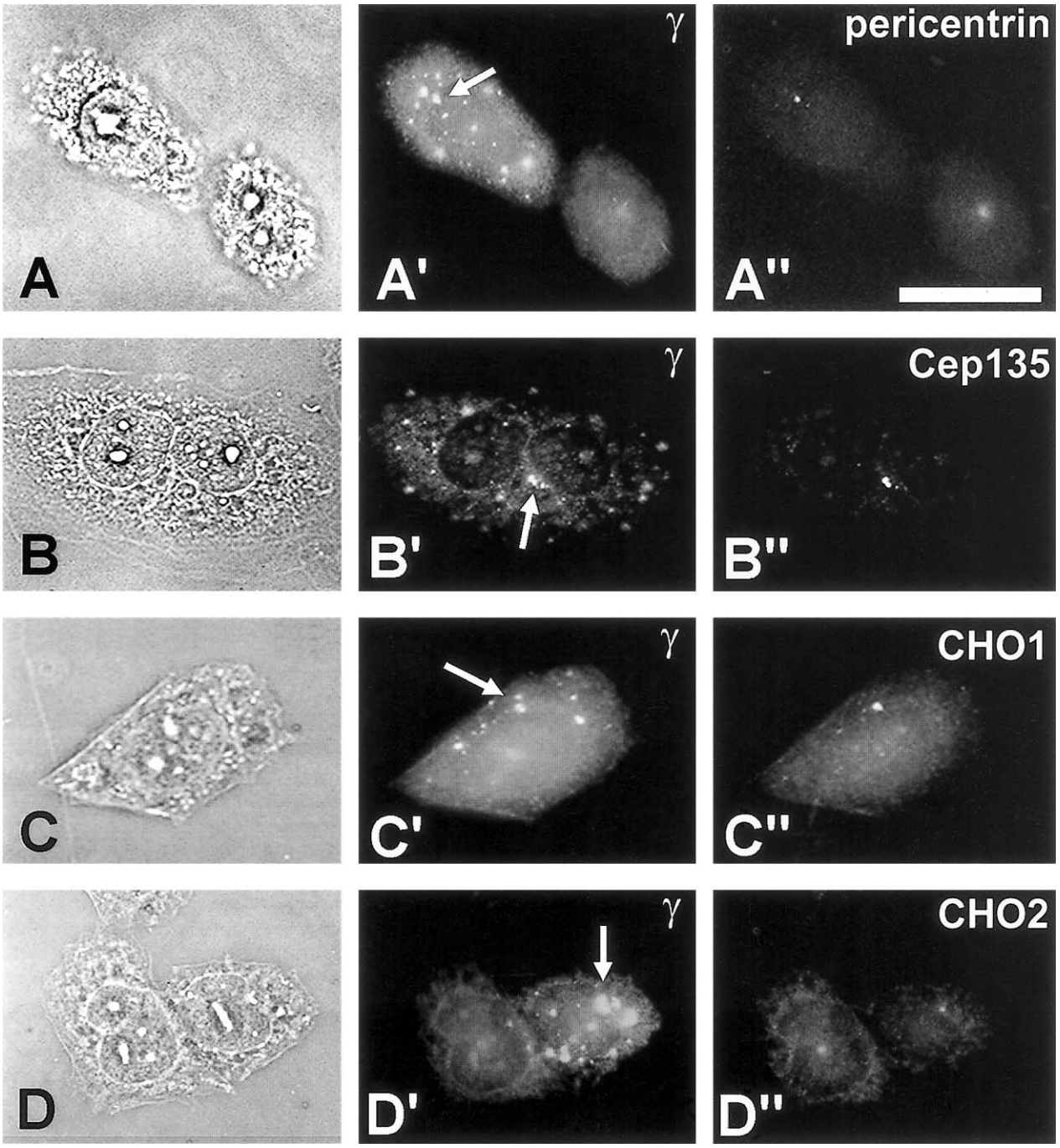

Fig. 4. Immunofluorescence staining of transfected CHO cells with centrosomal antibodies. The same cells were seen by phase-contrast (**A** to **D**) and fluorescence microscopy after double staining with polyclonal γ-tubulin antibodies (**A'** to **D'**) and either human autoantibody pericentrin (**A"**), mouse polyclonal Cep135 (**B"**), and mouse monoclonal CHO1/MKLP1 (**C"**), or CHO2 (**D"**). Cytoplasmic foci induced in the cytoplasm of cells overexpressing the full-coding CHO γ-tubulin sequence are not immunostained by the centrosomal antibodies. Arrows indicate the position of foci corresponding to the centrosome which are labeled by both γ-tubulin and other centrosomal proteins. Bar, 10 μm.

lin molecules are included in one μm of microtubules, it could be concluded that 2-3% of total tubulin proteins were decorated by immunogold particles. Since the level of non-specific background staining was low (Fig. 6C), it allowed us to carry out quantitative analysis of association of γ-tubulin with microtubules *in vitro*.

I) Interaction with Chlamydomonas flagellar microtubules: We prepared biochemical quantities of the monomeric form of γ-tubulin capable of co-sedimentation with brain microtubules *in vitro* (Vassilev *et al.*, 1995). Since the purified γ-tubulin was derived from *Chlamydomonas*, the assay of the binding capacity was done with flagellar microtubules prepared from the same species. *Chlamydomonas* axonemes were fairly constant in length (~2.5 μm) (Fig. 6A, C-E). When such ax-

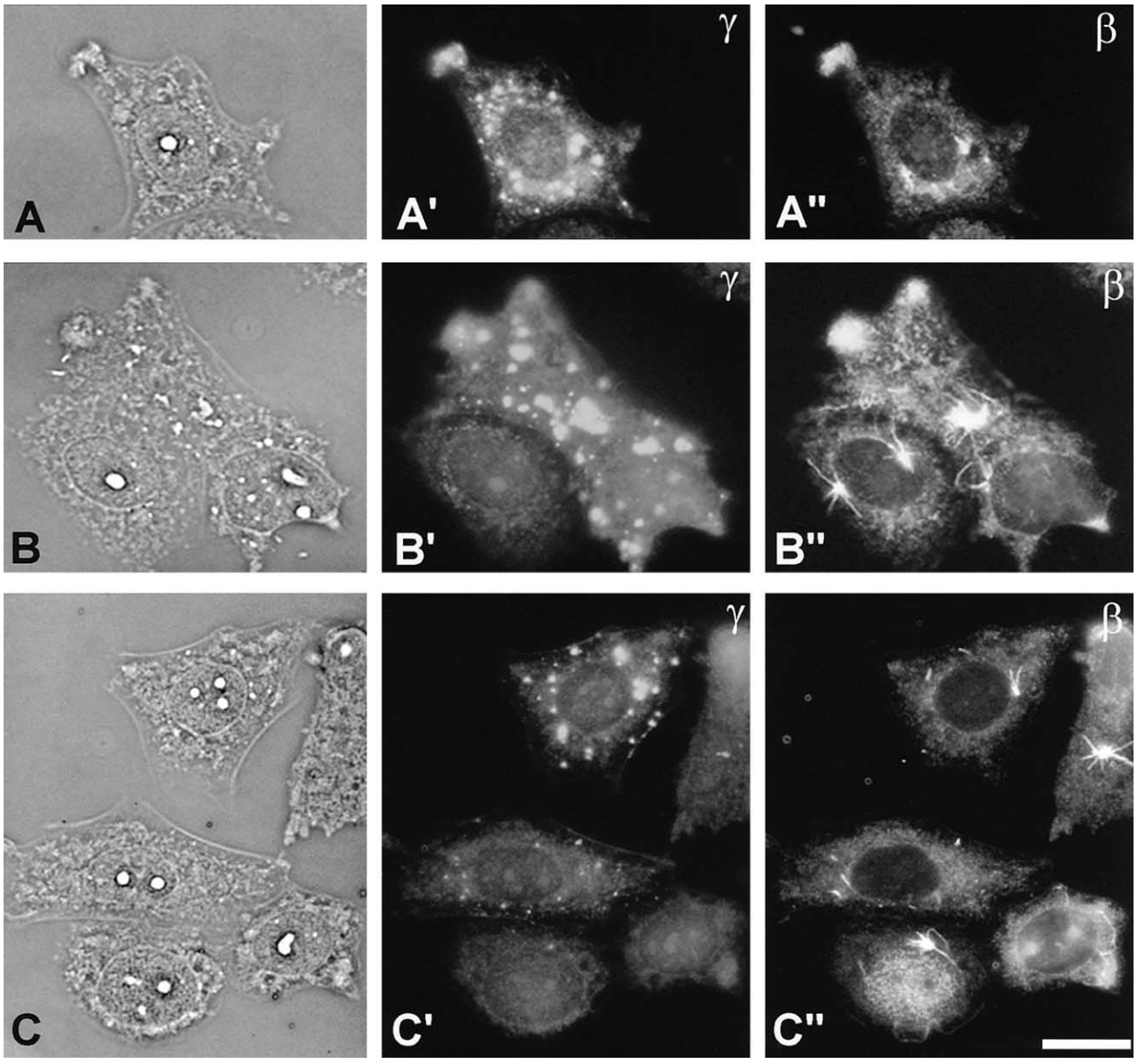

Fig. 5. Assembly of microtubules onto the centrosome upon removal of nocodazole. The same cells expressing the full-coding CHO γ-tubulin sequence were seen by phase-contrast (**A**-**C**) and fluorescence microscopy after double staining with polyclonal γ-tubulin (**A'**-**C'**) and monoclonal β-tubulin (**A"**-**C"**) antibodies. Cells were treated with nocodazole to depolymerize *in situ* microtubules, and microtubule formation was initiated around the centrosome upon removal of the drug in both transfected and non-transfected cells. Cells were fixed at 0.5 (**A**) and 2.0 (**B** and **C**) min after recovery from the nocodazole treatment at 37°C. The majority of cytoplasmic dots do not serve as the site for microtubule reassembly. Bar, 10 μm.

onemes were incubated with the purified γ-tubulin monomers in suspension, several gold particles were detected around the microtubules (D and E). Quantitative analysis showed that flagellar microtubules incubated with γ-tubulin decorated with an average of 3.8 particles (second column in Table 1). In controls in which no γ-tubulin was added, few randomly dispersed gold particles were detected on the background (Fig. 6C and first column in Table 1). Although the majority of particles were randomly distributed, we could detect several times more particles at the minus-end of microtubules (second column in Table 1; arrows in Fig. 6D and E). When the microscopic sample was prepared on grids (third column in Table 1), a more prominent association of γ-tubulin was detected with microtubules, both along the length and at their minus-end. An average 5.8 gold particles were counted in one axoneme, and every minus end was associated with about 0.9 particles.

The experiment with anti-α-tubulin antibody staining suggests 2-3% efficiency of detection of gold particles in our protocol (Fig. 6A, B). Assuming γ-tubulin binds to the minus-end of all protofilaments forming the "9 + 2" arrangement of flagellar microtubules, one can predict to find 5-7 gold particles at one side. It is, therefore, concluded that the specificity of γ-tubulin for the minus-end of microtubules would be consider-

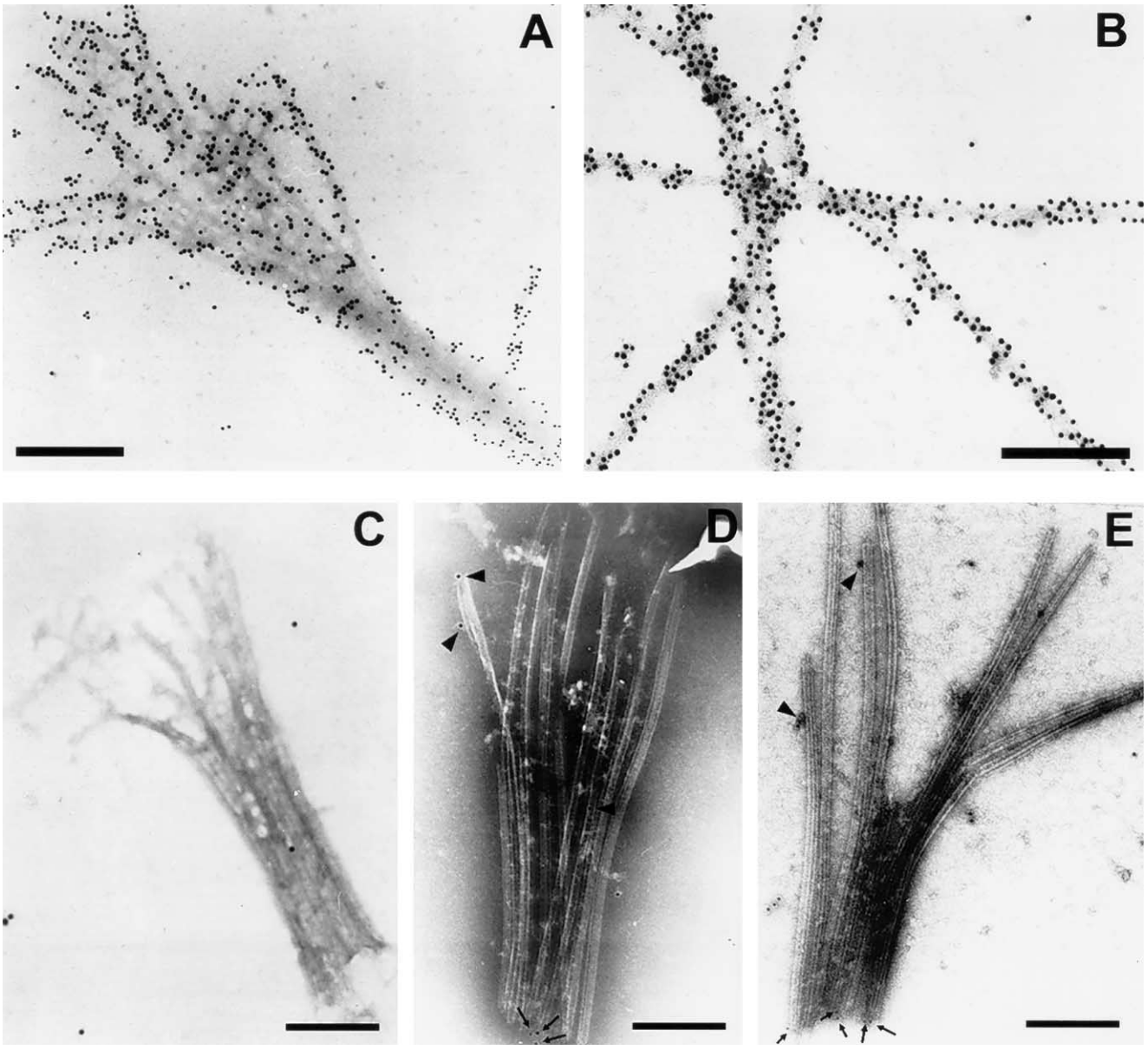

Fig. 6. Whole-mount electron micrographs of *Chlamydomonas* flagellar axonemes (**A**, **C**-**E**) and calf brain microtubules (**B**) immunostained with anti-α- (**A**, **B**) and anti-γ- (**C**-**E**) tubulin antibodies. Microtubules were incubated in suspension with (**D**, **E**) or without (**C**) γ-tubulin monomers purified from Sf9 cells expressing *Chlamydomonas* γ-tubulin as before (Vassilev *et al.*, 1995). While entire length of microtubules were decorated with α-tubulin probes (**A**, **B**), γ-tubulin staining reveals the presence of few particles on the microtubules (**D**, **E**). Particles bound to the wall and at the minus end of axonemal microtubules are indicated by arrowheads and arrows, respectively (**D** and **E**). Bars, 0.5 μm (**A**, **C**-**E**) and 0.2 μm (**B**).

ably low. Since we could detect higher rates of association in the samples prepared on electron microscopic grids, interaction of γ-tubulin with microtubules appears not to be intense enough to be maintained during pipetting procedures.

II) Interaction with reconstituted microtubules: Flagellar microtubules are in a stable form and directly nucleate from the basal body in a template manner. Although several reports indicate the presence of γ-tubulin in the basal body and/or centriolar structure (Muresan *et al.*, 1993; Liang *et al.*, 1996), it is unknown whether γ-tubulin possesses an intrinsic capability of binding to stable microtubules to control the organization of axonemal microtubules onto the basal body. In order to examine the interaction between γ-tubulin and microtubules, we next analyzed the direct association of γ-tubulin monomers with labile cytoplasmic microtubules known to be assembled from γ-tubulin-containing MTOC structures. Tubulin dimers active in polymerization into microtubules *in vitro* were prepared from *Chlamydomonas* flagellar outer doublets by sonication, then incubated with the purified γ-tubulin fraction. Results are summarized in Table 2, in which the distance between particles was calculated as total length of microtubules divided by a total number of associated gold particles. We

Table 1. Interaction of γ-tubulin with stable flagellar axonemes

	without γ-tubulin	with γ-tubulin in suspension	with γ-tubulin on grids
No of micrographs examined	14	25	13
No of axonemes analyzed	51	40	60
background GP*/100 μm^2	0.3 ± 0.14	1.0 ± 0.33	1.4 ± 0.72
No of GP/ axoneme	0.9 ± 0.21	3.8 ± 1.07	5.8 ± 1.89
No of GP/one plus end MT	0.25 ± 0.35	0.12 ± 0.10	0.14 ± 0.064
No of GP/one minus end MT	0	0.31 ± 0.18	0.93 ± 0.44

GP*: gold particles

Table 2. Interaction of γ-tubulin with lablile flagellar microtubules

	without γ-tubulin	with γ-tubulin in suspension	with γ-tubulin on grids
No of micrographs examined	8	10	13
No of MT#-end analyzed	107	124	527
total MT length (μm)	67	49	169
average MT length (μm)	0.6	0.4	0.3
background GP*/100 μm^2	0.2 ± 0.06	1.3 ± 0.69	1.4 ± 0.72
distance between GP in MTs (μm)	6.8 ± 3.00	3.4 ± 3.27	1.3 ± 0.85
No of GP/ one MT end	<0.01	0.01 ± 0.015	0.08 ± 0.025

MT#: microtubules
GP*: gold particles

could count one immunogold particle per every 1.3 μm of length. Since the average microtubule length used for the assy was 0.3-0.6 μm, only one out of two to four microtubules became decorated with immunogold particles. As in the case of flagellar axonemes, considerably more particles (third column in Table 2) were found to associate with microtubules incubated with γ-tubulin monomers on grids rather than in suspension (second column in Table 2). The protein's interaction with microtubule ends was also more prominent when γ-tubulin was incubated with microtubules which had already been placed on EM grids.

DISCUSSION

The primary amino acid sequence of γ-tubulin in CHO cells showed 97-98% homology to human and *Xenopus* γ-tubulin, confirming that this new member of the tubulin superfamily is a well-conserved MTOC/centrosome component. Overinduction of γ-tubulin resulted in the formation of various sizes of cytoplasmic foci in transfected CHO cells. A similar observation has been made by Shu and Joshi (1995) using monkey kidney COS cells which were transfected with the plasmid containing the full-coding human γ-tubulin sequence. Although both types of transfected cells contained similar cytoplasmic foci, the effects of the dots on the pattern of microtubule organization was different. While CHO cells expressing the full-coding and/or the truncated version of CHO γ-tubulin polypeptides failed to induce any foci that were capable of formation of microtubule assembly, the dots formed in the COS cell cytoplasm could serve as the center for ectopic nucleation of microtubules. In fact, such CHO cells retained normal arrays of microtubules, which is in sharp contrast to the COS

cells where the network of cellular microtubules was dramatically reorganized by overinduction of human γ-tubulin. Failure of γ-tubulin-containing dots to modulate the microtubule-organizing capacity in CHO cells is consistent with the observation that none of the centrosomal antigens examined so far were found to co-localize at the dots.

We can offer several reasons to explain the apparent discrepancy between the two experimental results. First, cDNA clones encoding γ-tubulin used for transfection were derived from different species (human vs. CHO). In light of the high degree of sequence homology between γ-tubulin in a wide range of organisms, however, the different sources of γ-tubulin molecules would be marginal. On the other hand, the difference in host cell lines used for transfection experiments may be important since Shu and Joshi (1995) have reported that tubule-like structures formed in the cell cytoplasm overexpressing γ-tubulin were seen only in COS cells, but not in HeLa, CHO or PtK_2 cells. COS cells might be more susceptible for modulation of γ-tubulin functions than other cultured mammalian cells.

γ-Tubulin is known to require the assistance of molecular chaperonin to attain its proper three-dimensional structure (Melki *et al*., 1993; Vassilev *et al*., 1995). It is, thus, important to consider the level of protein expression as well. Since the total amount of TCP1 available in cells would be limited, overinduction of γ-tubulin may result in accumulation of unfolded and/or improperly folded γ-tubulin in the cell cytoplasm. The higher the level of γ-tubulin, the more prominent the accumulation of non-functional molecules. Horio and Oakley (1994) reported the induction of human γ-tubulin in *Schizosaccharomyces pombe*: unlike transfected COS cells, no ectopic microtubule nucleation was detected in yeast transformants. Since the study in *S. pombe* showed a five-fold increase in γ-tubulin, whereas a 120-fold increase was reported in COS cells (Shu and Joshi, 1995), the difference in the formation of additional γ-tubulin-containing aggregates could be dependent on the level of protein induced in transfected cells. It is likely that the level of γ-tubulin expression in transformed CHO cells would be higher than that necessary for other molecules, including TCP1, to maintain γ-tubulin in a physiologically active state through their interaction with the γ-tubulin molecule. Thus, it is tempting to speculate that γ-tubulin-containing dots in the CHO cell could represent an inactive protein aggregates.

Since γ-tubulin is low in abundance and its localization is quite dispersed, general difficulties have been noted in the detection of γ-tubulin localization by immunoelectron microscopy. We faced a similar problem in our immunoelectron microscopic studies. Although we detected γ-tubulin attached to microtubules above the level of controls (Table 1 and 2), this association was not prominent enough to draw definite conclusions. Binding of γ-tubulin to microtubule ends also appears to not be extensive. While the molecule shows a several fold preference for the minus end of microtubules compared to the plus end, the majority of γ-tubulin was seen along the entire length of microtubules. These results contradict the kinetic as well as microscopic analyses of Li and Joshi (1995), who demonstrated specific interaction of γ-tubulin with the minus-end of microtubules *in vitro*. This may be due, in part, to the difference in the source of proteins used for labeling experiments. We employed purified γ-tubulin monomers from Sf9 cells, while Li and Joshi (1995) prepared isotope-labeled γ-tubulin which was transcribed and translated *in vitro* in reticulocyte lysate. Endogenous γ-tubulin has been shown to be present as a dimer (Vassilev *et al*., 1995) and to form a multicomplex of ring structures (Zheng *et al*., 1995). One possibility is that it is the γ-tubulin dimer/complex, and not the monomeric form of γ-tubulin, that can directly interact with microtubules in an end-specific manner. Since *in vitro* synthesized γ-tubulin molecules in the reticulocyte lysate are generally much lower in amounts than molecules produced in Sf9 cells, the protein could form a dimer/complex structure through binding to other MTOC-components available in the lysate.

The presence of γ-tubulin along the microtubule wall has also been detected in mitotic spindles in trypanosomas (Scott *et al*., 1996), plant (Liu *et al*., 1993) and animal cells (Gard, 1994; Lajoie-Mazenc *et al*., 1994). These observations allow us to speculate that localization of γ-tubulin at the minus-end of microtubules might be due to immobilization of the molecule in the centrosomal structure by interaction with other molecules included in the γ-tubulin complex and/or centrosome. Since *in vivo* association of γ-tubulin with microtubules has been detected only in mitotic cells (Liu *et al*., 1993; Lajoie-Mazenc *et al*., 1994), protein-protein interaction in the centrosomal structure is likely to be controlled in a cell cycle-dependent manner. Further biochemical and morphological analyses of γ-tubulin is hoped to reveal the dynamic nature of the centrosome.

ACKNOWLEDGMENT

This work was supported by grants to R.K. (CTR 3157R2 and Minnesota Medical Foundation) and A.V. (NSF RTG DBI9602237).

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(Received March 24, 1998 / Accepted May 6, 1998)