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A transient association of γ-tubulin at the midbody is required for the completion of cytokinesis during the mammalian cell division

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SUMMARY

γ-Tubulin, a relatively new member of the tubulin gene family, is localized primarily at the centrosome throughout the mammalian cell cycle and may play a key role in nucleation of cellular microtubule assembly. A transient association of γ-tubulin at the cytoplasmic bridge of telophase mammalian cells, the midbody, is recently documented. Using immunogold electron microsocopy and serial section reconstruction analysis, we show here that the transiently associated midbody γ-tubulin is localized at the minus ends of microtubules in the midbody structure. Using antisense RNA methods we also demonstrate that a selective depletion of transiently associated midbody γ-tubulin causes an abortive cytokinesis due to a failure in the morphogenesis of the midbody structure.

Key words: γ-tubulin, midbody, cytokinesis

INTRODUCTION

In most eukaryotic cells an ordered distribution of many cellular organelles, including the Golgi apparatus and the endoplasmic reticulum, is mediated by a highly organized radial array of tubular fibers, microtubules (Dabora and Sheetz, 1988; Kreis, 1990; Terasaki, 1990). In mammalian cells, microtubule assembly is initiated exclusively at the centrosome, a specialized organelle localized at the center of a cell (Osborn and Weber, 1976; Gould and Borisy, 1977; Brinkley et al., 1981). The centrosome has the properties of both nucleating microtubule assembly and anchoring the resulting microtubule arrays. Nucleation of microtubule assembly at the centrosome occurs with a distinct polarity: in most cells, the slow growing minus end of the microtubule remains attached to the centrosome and thus the rapidly growing plus end is distally disposed toward the cell periphery (Heidemann and McIntosh, 1980). During mitosis, the duplicated centrosomes form a bipolar microtubule array, the mitotic spindle, that is responsible for the correct segregation of chromosomes into nascent cells in karyokinesis (Bajer, 1972; Brinkley, 1985; McIntosh, 1991).

The final stage of mitosis is cytokinesis in which cytoplasm of a mother cell is divided into two daughter cells. During cytokinesis, a structurally complex intercellular bridge, the midbody, is formed (Mullins and Biesele, 1973; Mullins and McIntosh, 1979). Electron microscopic studies suggest that the midbody structure consists primarily of two bundles of microtubules that interdigitate at their plus ends at the center of the midbody structure (Euteneuer and McIntosh, 1980). In some species, such as Indian muntjac, the midbody structure can be resolved into seven zones that are characterized by distinct protein composition and ultrastructure characteristics (Rattner, 1992). So far, the biogenesis of the midbody microtubules is still not clear. It has been suggested that these microtubules could be remnants of interpolar microtubules of the mitotic spindle (Mullins and Biesele, 1977; Mastronarde et al., 1993). Alternatively, they could result from the transient nucleating activity of special microtubule organizing centers near the region where the midbody is formed (Julian et al., 1993).

γ-Tubulin is a relatively new member of the tubulin gene family with approximately 35% sequence identity to the classical α- and β-tubulins, while α- and β-tubulins share 35%-40% sequence identity with each other (Oakley and Oakley, 1989). Unlike α- and β-tubulins, which form microtubule polymers, γ-tubulin is excluded from the bulk of the microtubule lattice. Instead, γ-tubulin is enriched in mammalian centrosomes as well as in other microtubule organizing centers of distinct morphologies in many divergent species and cell types (Stearns et al., 1991; Horio et al., 1991; Joshi et al., 1992; Zheng et al., 1991; Baas and Joshi, 1992; Liu et al., 1993; for reviews, see Oakley, 1992; Joshi, 1993a, 1994). Using quantitative immuno-blot analysis, Stearns et al. (1991) found that γ-tubulin is present in mammalian cells at a low copy number of only 10^4 molecules per cell, less than 1% of the level of either α- or β-tubulin. Four lines of experimental evidence have implicated that γ-tubulin may be a key functional component of the centrosome and other microtubule organizing centers. First, disruption of the γ-tubulin gene in a filamentous fungus, *Aspergillus nidulans*, causes loss of mitotic spindles (Oakley et al., 1990); second, microinjection of a γ-tubulin antibody into mammalian cells disrupts microtubule nucleation by the centrosome and disrupts the morphogenesis of the mitotic spindle (Joshi et al., 1992); third, depletion of a γ-tubulin containing heteromeric protein complex from *Xenopus* egg extract also

diminishes the ability of the extract to support the formation of sperm mediated microtubule asters (Felix et al., 1994; Stearns and Kirschner, 1994). And finally, overexpression of γ-tubulin in a variety of mammalian cells causes γ-tubulin distribution throughout the cytoplasm where it forms copious microtubule nucleation sites (Shu and Joshi, 1995). These experiments provide compelling evidence for an important role of γ-tubulin in nucleation of microtubule assembly.

Recently, a transient association of γ-tubulin at the midbody was documented (Julian et al., 1993). Because γ-tubulin may play a key role in nucleation and organization of cellular microtubules, it is interesting to investigate the role of midbody-associated γ-tubulin in biogenesis of midbody structure and in completion of cytokinesis. In this study, we have determined the precise positional relationship between midbody microtubule ends and transiently localized γ-tubulin. Further, using antisense RNA methods we could deplete selectively the midbody associated γ-tubulin. This allowed us to determine the function of the midbody associated γ-tubulin. we demonstrate that the depletion of midbody γ-tubulin causes a failure in morphogenesis of midbody structure and an abortive cytokinesis.

MATERIAL AND METHODS

Vector construction

To construct plasmid RSV-anti-hγT, a full length human γ-tubulin cDNA was excised from KSII vector with *Eco*RI and a plasmid vector was excised from pRSV-β-globin (Gorman et al., 1982) with *Hin*dIII and *Bgl*II. The staggered ends were repaired by filling in the nucleotides with the Klenow fragment of DNA polymerase I. The cDNA was then ligated with the vector, and plasmids with the required antisense orientation of γ-tubulin cDNA with respect to the RSV promoter were selected.

Cell culture and transfection

Chinese hamster ovary (CHO), human fibroblast L cells, HeLa, and potoroo tridactylis kidney (PtK_2) cell lines were obtained from the American Type Culture Collection (Rockville, MD), human embronic kidney 293 cells were provided by Dr Gary Nabel of the University of Michigan Medical Center. All these cells were grown in DMEM medium (Sigma) with 10% fetal calf serum (Sigma) at 37°C in 5% CO_2. Cells in 100 mm dishes were transfected with 20 μg of plasmid DNA using the calcium phosphate precipitation procedure (Graham and van der Eb, 1973). For immunoblot analysis, cell extracts were prepared in 0.1 M Tris (pH 6.8) containing 1% SDS as previously described (Joshi et al., 1992). Protein concentrations were determined by a bicinochronic acid assay (Smith et al., 1985).

Gel electrophoresis and immunoblotting

Polyacrylamide gel electrophoresis for analyses of protein samples were performed as previously described (Laemmli, 1970). Proteins were electrophoretically transferred to nitrocellulose membrane (Schleicher & Schuell, Inc., Keene, NH) in one-half strength Laemmli gel running buffer containing 20% methanol. Typical transfer was for 4 hours at 50 V. Filters containing transferred samples were stained with Ponceau S (0.2% Ponceau S in 3% trichloroacetic acid) to identify the positions of molecular mass standards. Nonspecific protein binding was blocked by incubation in TBS-T blocking buffer (5% dry milk, 20 mM Tris, pH 7.6, 137 mM NaCl, 0.1% Tween-20) for 10 minutes. For immunological detection, γ-tubulin antibody (4 μg/ml) in TBS-T was allowed to react for 1 hour at room temperature. The filters were then washed five times (3 minutes each wash) in TBS-T. Horseradish peroxidase labeled goat anti-rabbit IgG secondary antibody in TBS-T was added and incubated for 1 hour at room temperature followed by five washes in TBS-T to remove unbound secondary antibody. Binding was detected by chemiluminescence (Western Light, Amersham) followed by autoradiography using Eastman Kodak Co. (Rochester, NY) XAR film. The relative protein levels were determined by densitometric analysis with a Dage-Mti densotometer and Image I Software (Universal Imaging Incorporation, west Chester, PA).

Antibodies

A rabbit polyclonal antibody specific to mammalian γ-tubulin was prepared by covalently crosslinking keyhole limpet haemocyanin with a 17 amino acid polypeptide, EEFATEGTDRKDVFFYC (the amino-terminal 16 residues of which are conserved among all known γ-tubulin sequences), followed by injection into rabbits. Characterization of this antibody was given in Joshi et al. (1992). Antibodies specific to α-tubulin and β-tubulin were purchased from Amersham Inc. The gold labeled antibodies were purchased from Janssen Biochimica (commercialized by Amersham Inc.). The fluorescent labeled antibodies were purchased from Cooper Biomedical, Inc.

Indirect immunofluorescence microscopy

For visualization of microtubules, γ-tubulin, and the nucleus, cells grown on glass were plunged into methanol at –20°C for 5 minutes. Cells were then rehydrated in phosphate buffered saline (PBS) and blocked in 1% BSA/PBS for 15 minutes. Primary staining were performed with 4 μg/ml γ-tubulin antibody and α- or β-tubulin monoclonal antibody (1:100 dilution) for 1 hour at room temperature. They were then rinsed with 1% BSA/PBS and either a fluorescein labeled anti-rabbit IgG antibody or a rhodamine-labeled anti-mouse IgG antibody was applied for 45 minutes at room temperature. The coverslips were rinsed 3 times with 1% BSA/PBS (DAPI was included in the final washing buffer) and mounted in Aquamount (Lerner Laboratories, New York). The cells were examined with a Zeiss Axiovert microscope.

Immunogold electron microscopy and serial section reconstruction

Chinese hamster ovary cells (CHO) and Ptk_2 cells were synchronized in S-phase by incubation for 24 hours in 2 mM thymidine, released by washing the thymidine for their synchronous progression into mitosis, fixed and permeabilized simultaneously with 2% gluteraldehyde and 0.5% Triton X-100 as described earlier (Joshi, 1993b). These cells were processed for γ-tubulin immunogold staining and flat embedding (Baas and Joshi, 1992). Cells embedded into a thin vapor of plastic were then visualized under a light microscope to locate cells in telophase, which were encircled, and sectioned using a Reichert Jung Ultracut S microtome. Serial sections (80-100 nm thick) were photographed in a JOEL electron microscope. To identify minus ends of microtubules we used our earlier published method (Joshi et al., 1986; Baas and Joshi, 1992). Sets of nine consecutive sections were

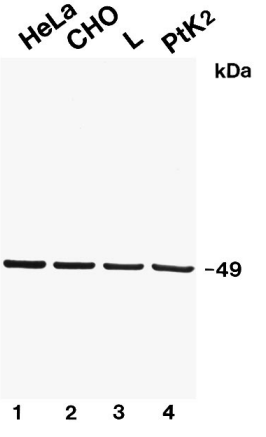

Fig. 1. Immunoblot analysis of γ-tubulin expression in divergent animal cell lines: 30 μg of total cell extracts from HeLa cells (lane 1), CHO cells (lane 2), L cells (lane 3), PtK_2 cells (lane 4) were loaded. The blot was probed by the γ-tubulin antibody and visualized by a chemiluminescent product (Western Light, Amersham).

aligned using the cellular boundaries surrounding the midbody structure as registration markers. A microtubule terminating in the middle section and absent from the next section was taken to be a true minus end (Baas and Joshi, 1992).

Microinjection

RSV-anti-hγT (0.5 μg/ml) alone or with 0.05 μg/μl RSV-β-gal (Shu et al., 1993) in the case of co-injections was injected using a Narashigi micromanipulator into nuclei of CHO and PtK_2 cells cultured on glass coverslips with a locator grid photo-etched by laser beam (Belco), according to a previously described method (Cappecchi, 1980). Sixty hours after injection, immunocytochemistry was performed as described above.

X-gal cytochemistry

To visualize expression of the reporter enzyme β-galactosidase, injected or transfected cells on glass coverslips were fixed in 1.25% glutaraldehyde/PBS for 5 minutes at room temperature, and washed twice with PBS. Cells were then stained in X-gal buffer (50 mM Tris-HCl, pH 7.5, 2.5 mM potassium ferrocyanide, 2.5 mM potassium ferricyanide, 15 mM NaCl, 1 mM $MgCl_2$, 0.5 mg/ml X-gal) for 2-4 hours.

In vitro transcription and translation

The in vitro transcription and translation was performed as described earlier (Z. Li and H. C. Joshi, unpublished). Briefly, two plasmids, GEM-hγT and GEM-anti-hγT were constructed by insertion of a 1,568 base pair fragment containing the complete human γ-tubulin cDNA into the *Eco*RI site of the pGEM vector in either sense or antisense orientation (Promega Biotech.) downstream of the T7 promoter. Sense and antisense γ-tubulin mRNA were then individually synthesized by T7 RNA polymerase using the two plasmids as template (Promega Biotech.). The translation reaction was carried out in a rabbit reticulocyte lysate system containing 60 μCi [^{35}S]methionine (Amersham) and 7 μg of sense γ-tubulin mRNA without or with a variable amount of antisense γ-tubulin mRNA. Following translation, the protein was subjected to two rounds of subsequent gel filtration chromatography on Sephadex G-25 columns to separate unincorporated amino acids. The translation products were electrophoresed in a sodium dodecyl sulfate polyacrylamide gel. Dried gels were autoradiographed for the visualization of the radioactive bands.

RESULTS

γ-Tubulin is transiently localized at the midbody

We have previously raised an epitope-directed rabbit polyclonal antibody specific for γ-tubulin (Joshi et al., 1992). To determine whether this antibody can detect γ-tubulin in divergent mammalian cell lines, we performed immunoblot analysis. We found that the γ-tubulin antibody recognized only a single 49 kDa band in all cell lines tested, including human HeLa and L cells, Chinese hamster ovary (CHO) cells, and PtK_2 cells (Fig. 1). These data suggest that all these cell lines have conserved γ-tubulin. We have also stained all of these cells with this γ-tubulin antibody. We found, as expected, that this antibody stained centrosomes during all phases of the cell cycle in all the cell lines tested (not shown). In addition, during the telophase of mitosis, the γ-tubulin antibody also stains a region around the minus ends of microtubule bundles at the midbody (Fig. 2A). The midbody staining by γ-tubulin antibody starts in early telophase and disappears in late telophase. These data are consistent with a recently documented study in which γ-tubulin was found to localize at the midbody for only 30-45 minutes (Julian et al., 1993).

Two-dimensional reconstruction analysis reveals γ-tubulin at minus ends of the midbody microtubules

Because microtubule bundles are major components of the midbody structure, the transiently associated γ-tubulin may play

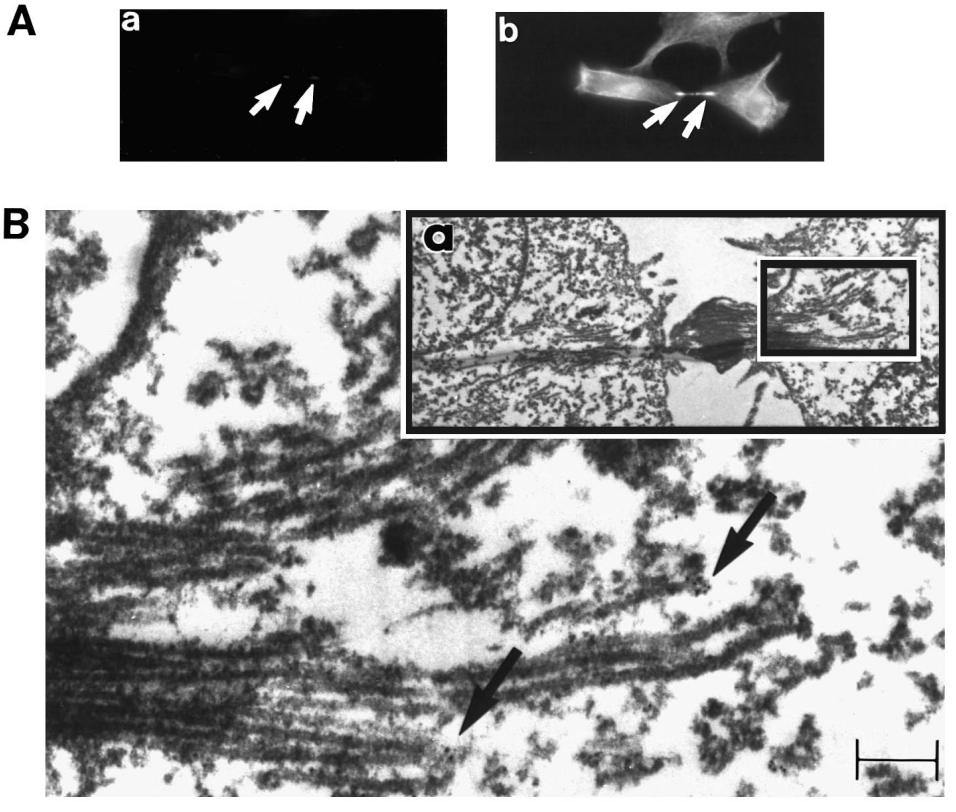

Fig. 2. Immunofluorescent and immunogold electron microscopic observation of γ-tubulin localization at the midbody. (A) γ-Tubulin is associated with midbodies of telophase cells as sugggested by immunofluorescent staining with a γ-tubulin antibody (a) and β-tubulin antibody (b) (arrows). (B) Immunogold electron microscopic observation of γ-tubulin localization at the midbody. γ-Tubulin is routinely found in the outer midbody zone reported to be enriched in minus ends of the midbody microtubules as illustrated by specific accumulation of gold particles (arrows). The inset shows a low magnification view of the midbody and the magnified area is demarcated by a box. Bar, 200 nm.

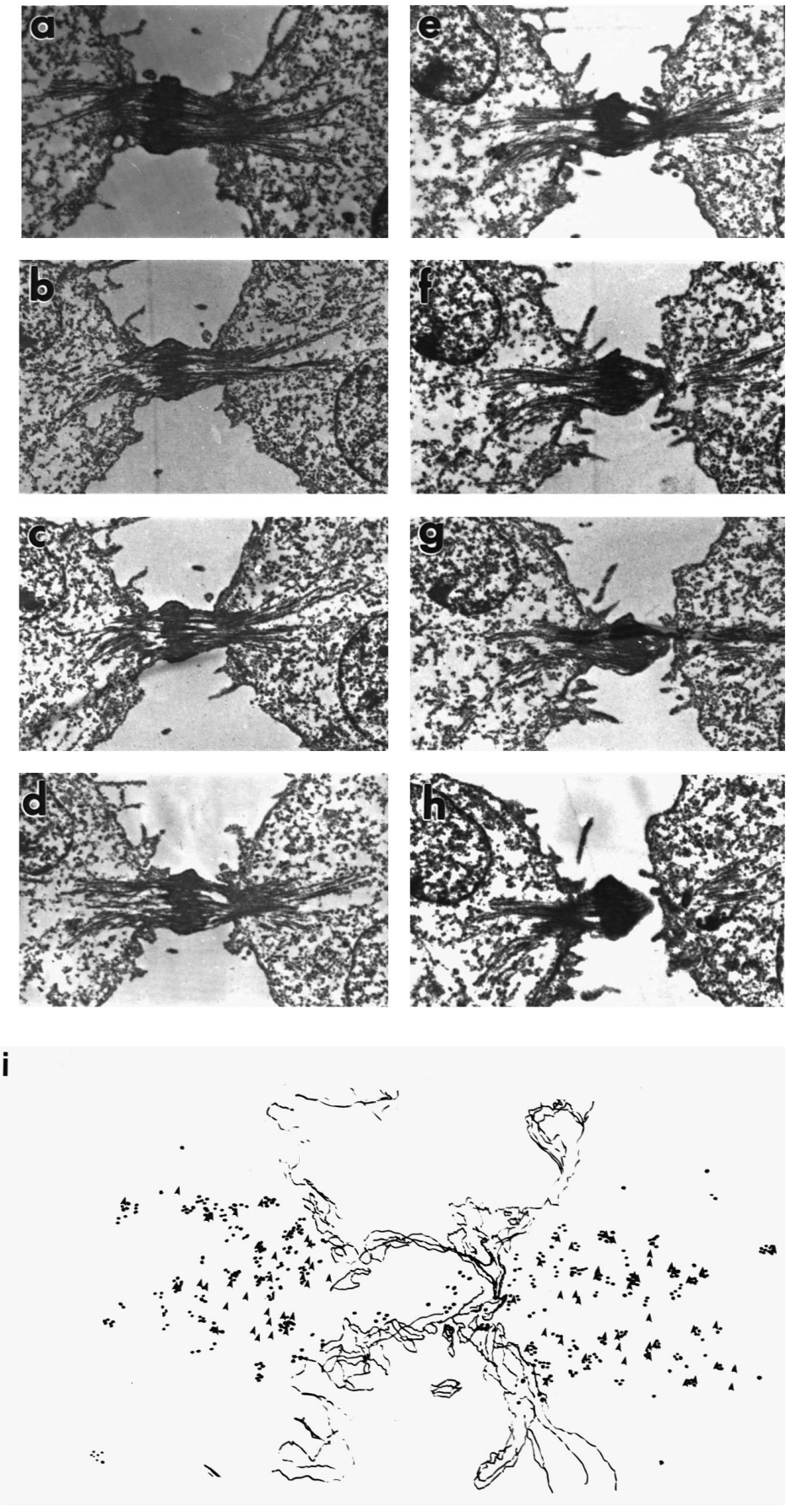

Fig. 3. Serial section reconstruction analysis of the midbody structure of a telophase cell. Eight serial sections of a midbody structure (a-h) were amplified for serial section reconstruction (i). The minus ends (arrowheads) of the midbody microtubules have been traced following previously described methods (Joshi et al., 1986; Baas and Joshi, 1992; Joshi, 1993). The relative position of γ-tubulin represented by gold particle clusters is shown (black dots). One dot represents at least four gold particles accumulated in the area of a dot. Fewer than four gold particles in the area of one dot is treated as background and neglected in the reconstruction analysis. The trajectories of individual microtubules were traced from eight consecutive sections. When presented in a single composite figure, the microtubule trajectories produced an extremely crowded image. Thus, for clarity, we present here only the ends of microtubules (arrowheads). A total of 5 telophase cells in which we could find gold particles were processed for serial section-reconstruction and consistent data were obtained.

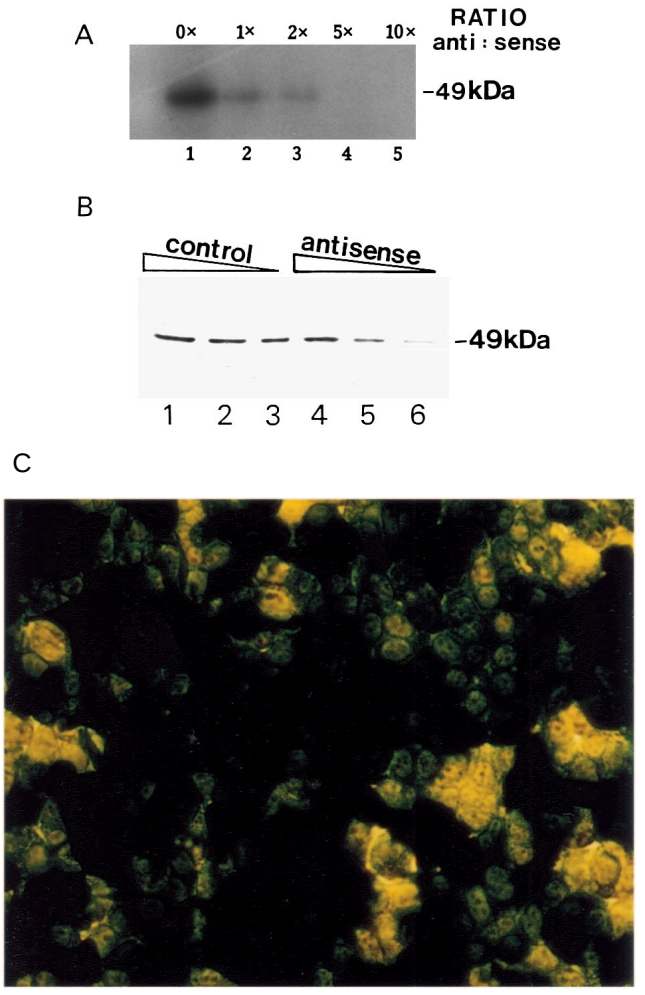

Fig. 4. Inhibition of γ-tubulin synthesis by antisense γ-tubulin mRNA in vitro and in vivo. (A) γ-Tubulin mRNA was synthesized and used in an in vitro translation system for the synthesis of [^{35}S]methionine labeled γ-tubulin either in the absence (A, lane 1), or in the presence of either 1× (lane 2), 2× (lane 3), 5× (lane 4), or 10× (lane 5) excess of the antisense RNA to the sense γ-tubulin mRNA. Note that a fivefold excess of antisense γ-tubulin mRNA blocks the synthesis of γ-tubulin from sense γ-tubulin mRNA. (B) γ-Tubulin synthesis was inhibited following transfection of an antisense γ-tubulin construct in vivo. At 48 hours after transfection with either RSV-anti-hγT or RSV-β-galactosidase construct, protein extracts were prepared and quantitated for western blot analysis using the γ-tubulin antibody. The samples were loaded with a serial dilution of 1.25-fold to ensure a measurement of bands in the linear range of detection by a laser scanning densitometer. (C) High transient transfection efficiency was achieved with 293 cells. 293 cells were transfected with the RSV-β-galactosidase construct by a calcium phosphate precipitation procedure and stained to visualize expression of β-galactosidase. In this panel, blue cells are transfected and yellow cells are untransfected.

a role in the morphogenesis of the midbody. To determine the precise localization of γ-tubulin within the midbody structure, we performed high resolution immunogold electron microscopy. We routinely found that γ-tubulin aggregates, as represented by gold particle clusters, were associated with putative minus ends of midbody microtubules in telophase cells (Fig. 2B, arrows). To determine if the gold particle clusters in fact lie in the same structural zone of the minus ends of midbody microtubules, we performed a two-dimensional serial section reconstruction analysis. In this analysis, by aligning microtubule trajectories from consecutive electron micrographs, we could track the ends of many microtubules. Using this method, we confirmed that the transiently associated midbody γ-tubulin, detected by the aggregates of gold particles, was localized within the zone of midbody enriched in minus ends of the midbody microtubules (Fig. 3). It should be noted that a subset of the minus ends have not been represented because of overlapping of microtubules in the serial sections along the vertical dimension. In late telophase cells, gold particles were restricted to the centrosomal regions (not shown). Although γ-tubulin was previously shown to be concentrated in this region, our data map precisely the minus ends of the midbody microtubules and their relationship with the γ-tubulin location. These data indicate an intimate relationship of γ-tubulin with minus ends of the midbody microtubules and are consistent with in vitro studies that demonstrate the interaction of γ-tubulin with microtubule minus ends (Z. Li and H. C. Joshi, unpublished).

Inhibition of g-tubulin synthesis by antisense g-tubulin mRNA in vitro and in vivo

The transient association of γ-tubulin at the minus ends of microtubules of the midbody may represent a consequence of recruitment of interpolar microtubule arrays along with their end-associated γ-tubulin at the midbody. Alternatively, a newly synthesized pool of γ-tubulin may be recruited at the midbody for nucleation of all or a subset of the midbody microtubules. In an effort to resolve this issue and dissect the role of the transiently associated midbody γ-tubulin, we attempted to use antisense RNA techniques to deplete the transiently associated midbody γ-tubulin. To do that, we first determined whether antisense γ-tubulin RNA can block synthesis of γ-tubulin in vitro. To do this, we used a recently developed in vitro system for the synthesis of radiolabeled γ-tubulin using γ-tubulin mRNA and [^{35}S]methionine (Z. Li and H. C. Joshi, unpublished). We found that an increasing molar ratio of antisense to sense mRNA diminished γ-tubulin synthesis (Fig. 4A), and a 5-fold excess of antisense RNA completely abolished synthesis of γ-tubulin in vitro (Fig. 4A, lane3). We conclude that antisense γ-tubulin RNA is effective in blocking synthesis of γ-tubulin by mammalian ribosomes in vitro.

To determine whether antisense γ-tubulin RNA can inhibit γ-tubulin synthesis in vivo, we constructed a plasmid RSV-anti-hγT, designed to express a high level of antisense γ-tubulin RNA from a strong eukaryotic promoter RSV-LTR (Gorman et al., 1982). Because the expression of antisense γ-tubulin RNA was lethal to cells and we could not establish a stable cell line even with a low basal-high inducible promoter (H.-B. Shu and H. C. Joshi, unpublished data) and the transient transfection efficiency being very low in the cell lines we used (HeLa, CHO, ptK$_2$, L cells) for the suitable cytological studies, it was difficult to do quantitative biochemical analysis of γ-tubulin depletion. To circumvent this problem, we explored many cell lines and found that human embryonic kidney cell line 293 yields a high transfection efficiency by the standard calcium phosphate precipitation procedure. Following transfection of a reporter construct, RSV-β-galactosidase into 293 cells, as many as 80% cells were transfected (Fig. 4C). This high transfection efficiency enabled us to confirm γ-tubulin depletion by

antisense γ-tublin RNA in vivo. We transfected the antisense construct RSV-anti-hγT into 293 cells and a quantitative western blot analysis was performed 48 hours after transfection. We found that the γ-tubulin level in the antisense construct transfected cells decreased approximately 30% in comparison to cells transfected with control plasmids (Fig. 4B), suggesting that γ-tubulin can be partially depleted by the antisense construct in mammalian cells.

Failure of midbody formation and cytokinesis following expression of antisense γ-tubulin RNA in mammalian cells

Because 293 cells have no prominent midbody structure, we did not analyze the phenotypic consequence of partial γ-tubulin depletion. Instead, we have used other cell lines for the study of phenotypic consequences of γ-tubulin depletion on the morphogenesis of the midbody structure and cytokinesis. To do this, we microinjected the antisense γ-tubulin construct, together with a reporter construct RSV-β-galactosidase (at a ratio 10:1) into the nuclei of PtK_2 or CHO cells cultured on glass coverslips that have a locator grid photo-etched by a laser beam. This procedure is a very effective means of transfection (Capecchi, 1980). The β-galactosidase expression plasmid served as a positive internal control for successful injection and resultant expression of injected DNA. Injection of this plasmid alone did not affect centrosomal or midbody γ-tubulin staining or progression of the normal cell cycle, but a co-injection of the antisense plasmid resulted in the absence of midbodies and accumulation of bi-nucleate cells (57% (35 out of 63) of β-galactosidase positive cells; Fig. 5). In many cases, daughter cells could not separate completely in the first round of the cell cycle, and remained connected by a cytoplasmic bridge instead of a normal midbody structure. We believe that this was a consequence of a partial depletion of the midbody associated γ-tubulin pool because such cells usually turned bi-nucleate after a subsequent round of nuclear division (Fig. 5h,i, arrows). Taken together, these data suggest that transient inhibition of new γ-tubulin synthesis causes failure of cytokinesis.

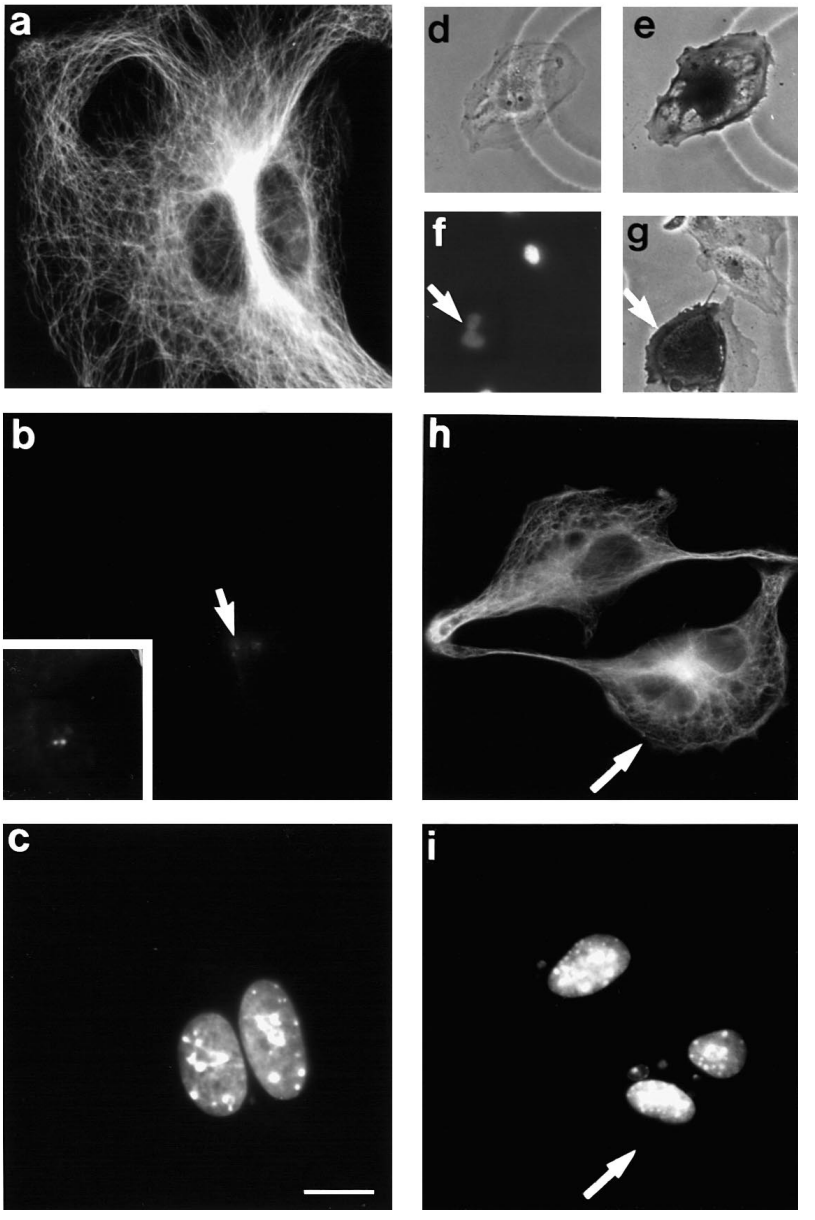

Fig. 5. Consequences of a transient γ-tubulin depletion on the mammalian cell cycle. (a-c) Phenotypic consequences of the in vivo inhibition of γ-tubulin synthesis. Microinjection of the antisense γ-tubulin construct leads to a failure of cytokinesis and results in the formation of a binucleate cell as visualized by DAPI staining in c. Injected cells had a substantial γ-tubulin staining (b, arrow, compare with the staining intensity of a control centrosome on the same coverslip in the inset) and well organized cytoplasmic microtubules (a). (d-g) Co-injections of RSV-anti-hγT and a reporter construct RSV-β-galactosidase. After developing a binucleate phenotype as seen by phase contrast microsopy (d), the expressed reporter enzyme was visualized using its chromogenic substrate, X-gal (e). A similar pair of X-gal cytochemistry and DAPI staining are shown in (g) and (f), respectively (note the quenching of DAPI florescence caused by the color of the X-gal reaction product (f, arrow). (h-i) Two daughter cells of an injected cell, one of which (arrow) showed a binucleate phenotype in the second round of the cell division, while the other completed this round successfully (one of the sister cells migrated away from the field of view). Bar, 10 μm.

In a widely accepted text book view of mammalian cell division, cytoplasm is divided by the cytokinetic furrow which assembles at the cell equator and contains cytoskeletal components, actin and myosin, as its major constituents (Darnell et al., 1989). In this context, it is puzzling that cytokinesis was disrupted due to partial depletion of γ-tubulin, a protein important for organization of microtubules. To resolve this paradox, we wished to observe, in real time, the entire cell division process of cells deprived of new γ-tubulin synthesis. HeLa cells, grown on glass coverslips with a locator grid, were injected into nuclei with an RSV-anti-hγT construct and monitored as they progressed through the cell cycle by phase contrast microscopy (Fig. 6). These cells formed a furrow at the correct location which attempted to pinch the cellular cytoplasm in the midzone (Fig. 6B). However, this was not followed by the morphogenesis of a midbody. Instead, the constriction process was subsequently reversed by a slow dilation leading to disappearance of the constricted zone and formation of a bi-nucleate cell (Fig. 6C).

DISCUSSION

A previous study suggested that γ-tubulin is associated transiently with the midbody in mammalian cells and formation of midbody can be blocked by the injection of a γ-tubulin antibody (Julian et al., 1993). In this study, we have further explored the function of the transient associated midbody γ-tubulin. Using high resolution immunogold electron microscopy and two-dimensional serial section reconstruction analysis, we show that γ-tubulin is localized at the zone enriched in minus ends of the midbody microtubules in telophase mammalian cells. Depletion of the transiently associated midbody γ-tubulin blocks formation of midbody and results in failure of cytokinesis.

The simplest model that emerges from these data is that, in the absence of new synthesis, γ-tubulin is not available for a cell cycle dependent assembly of the midbody microtubules and these cells fail to carry out cytokinesis. As a result, the two newly divided daughter nuclei are trapped in the mother cell. New γ-tubulin synthesis during each cell cycle may not be as important, in the time frame of our experiment, for a more stable pool of γ-tubulin that is associated with the centrosome throughout the cell cycle. This might account for our observation that in the time frame of our experiment (60 hours after microinjection), the centrosomal γ-tubulin was not completely depleted (although we did observe weaker staining when compared with that of the uninjected cells, see Fig. 5b). In the injected cells the cellular microtubules could nucleate from centrosomes (Fig. 5a-c). These data might suggest that antisense γ-tubulin RNA expression had little effect on the abundance of the centrosomal γ-tubulin and thus on the centrosome function. It is possible that the γ-tubulin enriched in centrosome might have a long half life because of its association with other centrosomal components. Furthermore, the bi-nucleate cells might die before the complete depletion of γ-tubulin.

What is the mechanism of γ-tubulin involvement in cytokinesis? Several studies have shown that γ-tubulin may be a protein responsible for the nucleation of microtubule assembly (Oakley et al., 1990; Horio et al., 1991; Joshi et al., 1992; Felix et al., 1994; Stearns and Kirschner, 1994; Shu and Joshi, 1995). In light of these observations, the midbody γ-tubulin may be involved in formation of a transient nucleation center for the assembly of the midbody microtubules, the major cytoskeleton in the midbody (Mullins and Biesele, 1973; Mullins and McIntosh, 1979). As an alternative, it has been suggested that spindle microtubules may be disassociated with the polar regions of the mitotic spindle and then translocated to constitute the midbody (McIntosh and Landis, 1971; McIntosh et al., 1975; Mastronarde et al., 1993). Taken together, these data

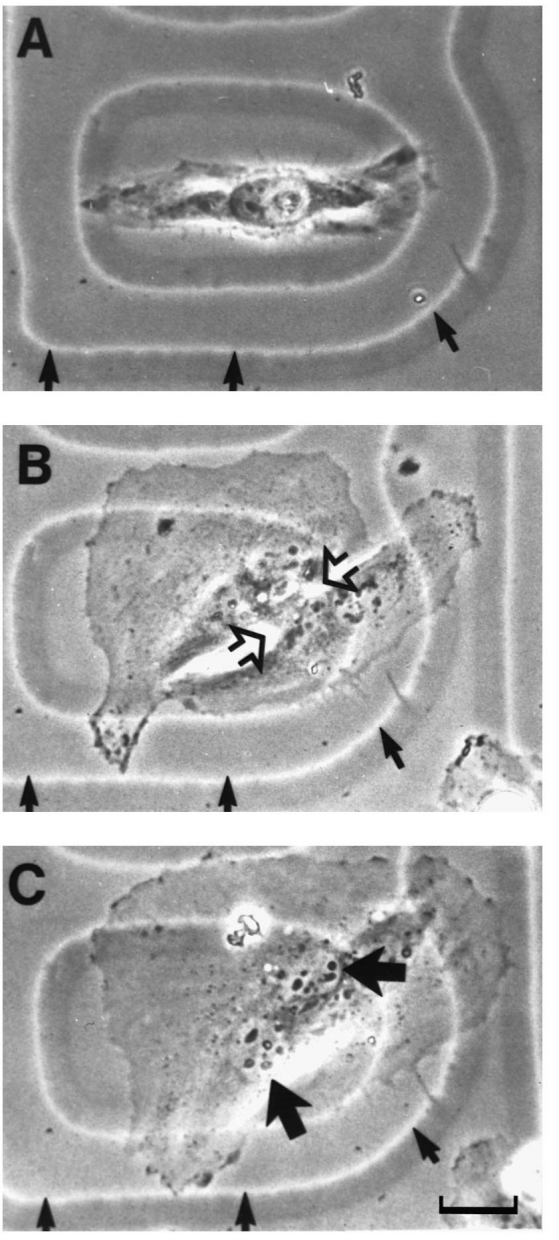

Fig. 6. A cell undergoing an abortive cytokinesis due to the expression of the γ-tubulin antisense RNA. The injected cell in the interphase (A), was followed under phase contrast optics. The cell constricted the cytoplasm at telophase approximately 12 hours after injection (open arrows) (B). Instead of dividing the cell into two daughters, the constricted zone dilated and the two daughter nuclei ended up in a common cytoplasm of the large cell 24 hours after injection (large filled arrows) (C). The photoetched pattern of the locator grid is seen in all panels (small filled arrows). Bar, 10 μm.

suggest that the spindle microtubules at the interzone might not be sufficient to account for the midbody microtubules, and a newly nucleated population of microtubules by the transiently associated midbody γ-tubulin might be required for formation of a functional midbody structure and successful completion of cytokinesis.

Our results are consistent with one study in the fission yeast *S. pombe* (Horio et al., 1991). In *S. pombe*, cytoplasmic microtubules are nucleated from microtubule organizing centers located in the division plate which represents a homologous structure of the midbody in mammalian cells (Hagan and Hyams, 1988). It has been shown that γ-tubulin is localized at this structure in the division plate and is responsible for nucleation of cytoplasmic microtubules in *S. pombe* (Horio et al., 1991). Further, the disruption of γ-tubulin gene inhibits cytokinesis in *S. pombe* (Horio et al., 1991). Taken together, our and other's data suggest that transient nucleation of microtubules at the midbody of mammalian cells or the division plate of *S. pombe* by γ-tubulin is required for successful completion of cytokinesis. However, the mechanism by which microtubules in the midbody or division plate are involved in cytokinesis is still an open issue.

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