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Dictyostelium γ-tubulin: molecular characterization and ultrastructural localization

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SUMMARY

The centrosome of *Dictyostelium discoideum* is a nucleus-associated body consisting of an electron-dense, three-layered core surrounded by an amorphous matrix, the corona. To elucidate the molecular and supramolecular architecture of this unique microtubule-organizing center, we have isolated and sequenced the gene encoding γ-tubulin and have studied its localization in the *Dictyostelium* centrosome using immunofluorescence and postembedding immunoelectron microscopy. *D. discoideum* possesses a single copy of a γ-tubulin gene that is related to, but more divergent from, other γ-tubulins. The low-abundance gene product is localized to the centrosome in an intriguing pattern: it is highly concentrated in the corona in regularly spaced clusters whose distribution correlates with the patterning of dense nodules that are a prominent feature of the corona. These observations lend support to the notion that the corona is the functional homologue of the pericentriolar matrix of 'higher' eukaryotic centrosomes, and that nodules are the functional equivalent of γ-tubulin ring complexes that serve as nucleation sites for microtubules in animal centrosomes.

Key words: Centrosome, γ-Tubulin, Immunoelectron microscopy

INTRODUCTION

Many cellular activities depend on a precisely organized and dynamic system of microtubules. Their number, polarity, and three-dimensional organization are controlled by organizing centers, the best known being the centrosome, a centrally located body where microtubules are anchored with their minus ends (for reviews, see Brinkley, 1985; Kalnins, 1992; Kellogg et al., 1994; Balczon, 1996). The structure of centrosomes from phylogenetically diverse organisms may be quite heterogeneous. A typical mammalian centrosome consists of a pair of centrioles surrounded by an amorphous, electron-dense matrix, the pericentriolar material, from which microtubules emerge (Porter, 1966; Snyder and McIntosh, 1975; Gould and Borisy, 1977; Kellog et al., 1994). In budding yeast, on the other hand, the centrosome (termed spindle pole body) is a multilayered structure embedded partially into the nuclear envelope (Byers and Goetsch, 1975; Snyder, 1994). Centrosomes lacking centrioles are also prevalent among filamentous fungi, plant cells, protozoa, and slime molds where their morphology may range from that of a compact body to a diffuse or fuzzy zone near the cell center.

Despite their structural diversity, centrosomes share homologous proteins (Kalt and Schliwa, 1993). One of the best-studied to-date is γ-tubulin, a relatively new member of the tubulin superfamily. γ-Tubulin was initially identified as a suppressor of a β-tubulin mutation in *Aspergillus* (Oakley and Oakley, 1989) and has since been found in many other eukaryotes, including fungal, protozoan, plant, and animal species (for reviews see Joshi, 1994; Burns, 1995a), suggesting that γ-tubulin is important for centrosome function. This conclusion is supported by the fatal inhibition of nuclear division and cell cycle progression after disruption of the gene (Oakley et al., 1990; Horio et al., 1991; Sunkel et al., 1995; Spang et al., 1996) and the failure of microtubule assembly in the presence of specific antibodies (Joshi et al., 1992; Julian et al., 1993; Ahmad et al., 1994; Stearns and Kirschner, 1994). γ-Tubulin is concentrated at microtubule-organizing centers where it associates specifically with microtubule minus ends (Li and Joshi, 1995). It appears to be part of a larger, ring-shaped complex, the γ-tubulin ring complex, or gammasome, that includes at least seven other polypeptides (Zheng et al., 1995; Moritz et al., 1995: Erickson and Stoffler, 1996). In addition, a sizeable pool of γ-tubulin is dispersed in the cytoplasm, presumably in the form of gammasomes (Raff et al., 1993; Marschall et al., 1996; Moudjou et al., 1996), that can be recruited to the centrosome to enhance nucleation (Ohta et al., 1993; Felix et al., 1994; Stearns and Kirschner, 1994). These findings suggest that γ-tubulin can shuttle between the centrosome and cytoplasm, perhaps in response to regulatory signals involving the phosphorylation of centrosomal components (Masuda et al., 1992; Ohta et al., 1993; Stearns and Kirschner, 1994). Based on immunofluorescence microscopy, γ-tubulin has also been suggested to associate with microtubules along their length, particularly in highly ordered microtubule arrays such as the mitotic spindle, the midbody,

and the plant cell phragmoplast (Liu et al., 1993, 1994; Lajoie-Mazenc et al., 1994; Sunkel et al., 1995). Overexpression of γ-tubulin in mammalian cells also induces the formation of novel tubular structures (Shu and Joshi, 1995), perhaps in association with the wall of pre-existing microtubules. The functional importance of these associations is unclear.

Among acentriolar centrosomes, the microtubule-organizing center of amoebae of the slime mold *Dictyostelium discoideum* stands out as a paradigm of a highly organized, morphologically well defined body responsible for the organization of the cell's 30-40 interphase microtubules. It is a more or less box-shaped structure consisting of three major layers that form an electron-dense core, which in turn is covered by an amorphous coat, the corona, from which microtubules radiate into the cell periphery (Moens, 1976; Kuriyama et al., 1982; Omura and Fukui, 1985). To relate the function of γ-tubulin in microtubule nucleation to the molecular architecture of this unique centrosome, we have undertaken a molecular characterization of *Dictyostelium* γ-tubulin and have studied its association with the centrosome at both the light and electron microscopic level. We demonstrate a localization of γ-tubulin to discrete substructures of the centrosome that implies a specific role in the deployment of microtubules in *Dictyostelium* amoebae.

MATERIALS AND METHODS

Molecular cloning of *D. discoideum* γ-tubulin

Degenerate primers in conserved regions of γ-tubulin generously provided by T. Stearns (TGA.1 and TGA.4; Stearns et al., 1991) were used to generate a fragment of the appropriate length using genomic DNA of *D. discoideum* (kindly provided by A. Noegel) as a template. The PCR product was cloned into pBluescript II SK+ (Stratagene, Heidelberg, Germany) and sequenced by the dideoxy chain termination method (Sanger et al., 1977). It was found to be a γ-tubulin gene fragment. Since the screening of several *D. discoideum* cDNA libraries using this fragment was unsuccessful, the PCR fragment was used to screen a partial genomic library of *Eco*RI/*Nde*I-digested DNA (generous gift of A. Hofmann) constructed in pUC19. The genomic fragment obtained lacked ~400 bp at the 3′-end, which were obtained by RACE-PCR (Frohmann et al., 1988). To this end, mRNA was purified from total RNA with the Oligotex-dT poly(A)$^+$ kit (Qiagen, Hilden, Germany) and transcribed into cDNA using the Stratagene RT-PCR kit. The final γ-tubulin cDNA sequence was obtained by PCR using linker primers extended by appropriate restriction sites, which were used to clone the cDNA insert into pBluescript SK.

For Southern blots, *D. discoideum* genomic DNA was prepared as described elsewhere (Noegel et al., 1985). Hybridization was at 37°C overnight in 2× SSC containing 50% formamide. For northern blots, RNA was isolated, electrophoresed, and blotted as described previously (Noegel et al., 1985).

Expression of *D. discoideum* γ-tubulin and antibody production

The γ-tubulin cDNA was cloned into the pMALc2 vector and transformed into electrocompetent *E. coli* TB-1 cells. In this vector γ-tubulin is expressed as an N-terminal fusion with maltose binding protein (~43 kDa) under control of the IPTG-inducible tac promotor. Protein expression was at 21°C overnight on a rotary shaker. Cells were harvested and lysed by freeze-thawing and sonication. After sedimentation of cell debris the cell extract was supplemented with 200 mM NaCl (final concentration), and the fusion protein was purified by affinity chromatography on amylose agarose (NEB, Schwalbach, Germany) according to the manufacturer's instructions. The fusion protein was electrophoresed on a preparative SDS-polyacrylamide gel. 0.4 mg of the γ-tubulin MBP fusion protein were recovered by electroelution, dialysed against 0.2 M $NaHCO_3$/0.02% SDS, lyophilized, and used for custom immunization of a rabbit (Charles River, Kisslegg, Germany).

For antibody purification, 3 mg of the γ-tubulin-MBP fusion protein were cleaved at the fusion site with 30 µg factor Xa protease and electrophoresed on a preparative SDS gel. The γ-tubulin band was excised, recovered by electroelution, and coupled to a NHS-Hi Trap column (Pharmacia, Freiburg, Germany) according to the manufacturer's instructions except that 0.1% SDS was added to keep the recombinant γ-tubulin soluble. From 1 ml of the rabbit serum approx. 50 µg of antibodies were eluted using 100 mM glycine-HCl, pH 2.7. Since about 50% of the eluted antibodies were still binding to MBP in western blots, a column was prepared using MBP, and the eluate from the first column was re-chromatographed. The flow-through contained only antibodies against γ-tubulin, as determined by immunoblots.

Cell culture and immunofluorescence microscopy

D. discoideum wild-type strain AX2 was grown as described (Claviez et al., 1982). For immunofluorescence microscopy, cells were allowed to settle and spread onto clean coverslips. They were fixed for 1 minute with 2% paraformaldehyde and 0.05% glutaraldehyde in low ionic strength PHEM buffer (15 mM Pipes, 5.25 mM Hepes, 10 mM EGTA, 0.5 mM $MgCl_2$, pH 6.9; Schliwa and van Blerkom, 1981) supplemented with 0.02% NP40, followed by 30 minutes of postfixation with 2% paraformaldehyde in the same buffer. Primary antibodies were applied for 40 minutes and secondary antibodies (Dianova, Hamburg, Germany) for 30 minutes, all diluted in phosphate-buffered saline containing 0.1% BSA. DNA was labeled with DAPI at a final concentration of 1 µg/ml for 1 minute. Cells were viewed in a Zeiss Axiophot microscope equipped with a Super Rollex Camera (Linhof, München, Germany) and photographed on Kodak TMAX 400 film.

Preparation of protein extracts, gel electrophoresis, and western blotting

For total cell extracts, suspension cultures of *D. discoideum* at a density of approx. 5×10^6 cells/ml were harvested and washed three times with 17 mM phosphate buffer, pH 7.0. 50 µl of cell suspension containing 2×10^7 cells were mixed with an equal volume of lysis buffer (9 M urea, 10% SDS, 5% 2-mercaptoethanol) and boiled for 3 minutes.

Nuclei were isolated essentially as described by Kuriyama et al. (1982). After sedimentation of nuclei, 50 µl of the supernatant and 50 µl of the resuspended nuclear pellet (each corresponding to the material from 2×10^7 cells) were mixed with lysis buffer, boiled, and used for SDS gel electrophoresis.

SDS acrylamide gel electrophoresis (T=12.5%/C=0.5%) was performed as described by Bollag et al. (1996). Proteins were blotted onto nitrocellulose membranes by the semidry procedure using the buffer system of Kyhse-Anderson (1984), modified by the addition of 20% methanol. Immunostaining was carried out according to the method of Bollag et al. (1996) using the calf intestine alkaline phosphatase reaction with bromo-chlor-indolyl phosphate/nitro blue tetrazolium chloride.

Electron microscopy

Epoxy embedding

Cells on glass coverslips were fixed with 0.5% glutaraldehyde in PHEM buffer (30 mM Pipes, 12.5% mM Hepes, 4 mM EGTA, 1 mM $MgCl_2$) supplemented with 0.5% Triton X-100 for 15 minutes. Postfixation, dehydration, and en-bloc staining with uranyl acetate were carried out according to standard procedures.

Lowicryl embedding

Cells were allowed to spread on plastic coverslips (Thermanox, Nunc, Naperville, USA) and fixed as described above. Dehydration and embedding in Lowicryl were carried out in an Automatic Freeze-Substitution System (AFS, Leica, Heerbrugg, Switzerland) essentially according to the procedure outlined by Robertson et al. (1991). Sectioning was done on either Reichert Ultracut E or Ultracut S microtomes (Leica). Sections were labeled with either rabbit anti-γ tubulin or rabbit anti-GFP antibody (Clonetech, Heidelberg), followed by secondary antibodies coupled to 6 nm colloidal gold (Aurion, Wageningen, Netherlands) or 5 nm Protein A-gold (J. W. Slot, Utrecht University, Netherlands). They were stained with uranyl acetate and lead citrate and viewed in either a JEOL 1200C or Philips CM120 electron microscope.

RESULTS

Molecular characterization of γ-tubulin

A combination of PCR, genomic cloning, and 3′-RACE was used to clone the *D. discoideum* γ-tubulin gene, as described in Materials and Methods. The open reading frame of *D. discoideum* γ-tubulin was 1,389 bp in length and encodes a protein of 462 amino acids with a predicted molecular mass of 52.234 (Fig. 1). The gene has an intron at base position 49, a position that is shared with other γ-tubulin genes (e.g. those of *Schizosaccharomyces pombe*, *Physarum falciparum*, and *Aspergillus nidulans*; Stearns et al., 1991; Oakley and Oakley, 1989; Lajoie-Mazenc et al., 1996). The southern blot analysis suggests that only one gene is present in *D. discoideum* (Fig. 2). Northern blot analysis demonstrated a message of about 1.5 kb which is present in very low abundance in exponentially growing cells as well as during the first 6 hours of *D. discoideum* development (not shown).

D. discoideum γ-tubulin is between 60% and 75% identical to γ-tubulins of a variety of other species from the plant, animal, and fungal kingdoms. An exception is the putative γ-tubulin homologue of *S. cerevisiae*, TUB4p (Sobel and Snyder,

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MPREIITLQVGQCGNQIGSEFWKQLCKEHGINAAGYLEEF  40
AKPGIDRKDVFFYQSDDDHYVPRALLLDLEPRVIDSIKTS  80
EYSGLYNQENIFVAEKGTGAGNNWANGYKQGESFYDDIFD 120
MIDREADGSESLEGFLLTHSISGGTGSGMGSYILERLNDR 160
FPKKIIQTYSVFPDDSSVVVQQYNSVLTLKRLIENADSTV 200
VLDNNALHRIVGENLRIDQPTMDQTNSLVSTVMSASTTTL 240
RYPGYMNNDLVGMLASLIPTPKCHFLITGYTPLSIDRQTE 280
SVRKTSVLDVMRRLLQPQNIMVSAPTKTGKYISILNIIQG 320
DVDPTQIHNSLQRIREKRMATFIDWGPASIQIALSKKSPY 360
IKSSHKVSGLMLANHTSVNHLFSHIIQQYDKVRKKQAFLA 400
NYTRESGEILQEFDIAREMLDDLVQEYKAAEQSDYINYHM 440
NKEFNNNNNNNNGNPQYQYGTV*                   463
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Fig. 1. Deduced amino acid sequence of *Dictyostelium discoideum* γ-tubulin. The positions of the primers used in the initial PCR experiment are underlined, and their direction is indicated by arrows.

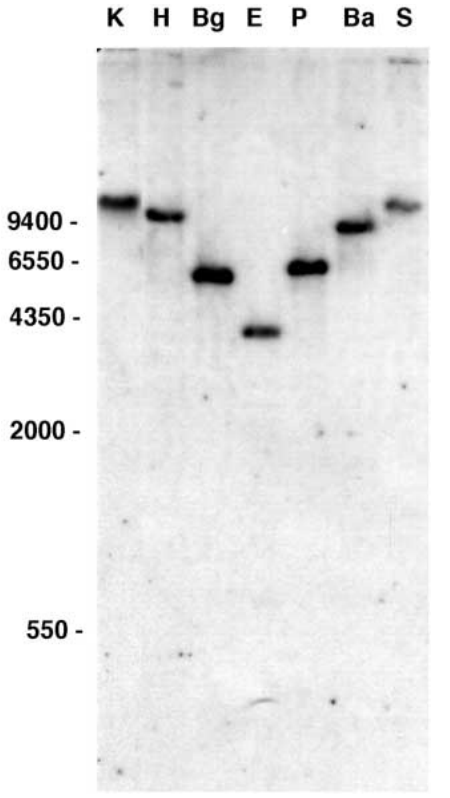

Fig. 2. Southern blot analysis of *Dictyostelium discoideum* genomic DNA digested with *Kpn*I (K), *Hin*dIII (H), *Bgl*I (Bg), *Eco*RI (E), *Pst*I (P), *Bam*HI (Ba), and *Sph*I (S) and probed with the PCR fragment (aa 49-321). Molecular sizes are shown in bp on the left. The patterns observed are consistent with the presence of only one gene for γ-tubulin.

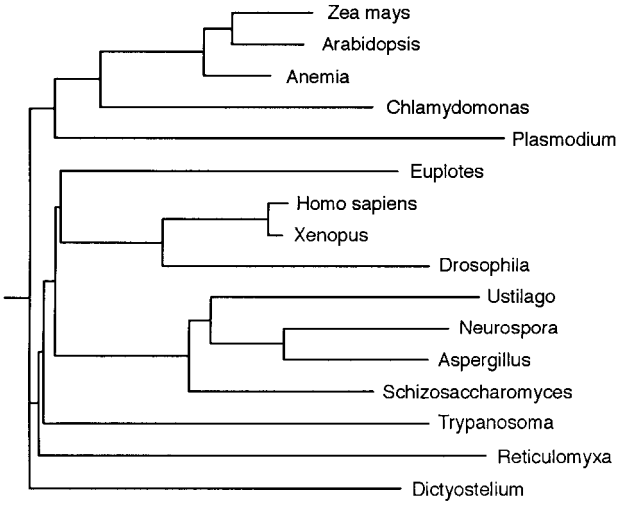

Fig. 3. Phylogenetic tree of γ-tubulins calculated with the program PROTDIST. The resulting distance matrix was used as input for the FITCH program, which estimates a phylogeny, and plotted as a phenogram using DRAWGRAM. All three programs belong to the PHYLIP package. Accession numbers: *Zea* X78891, *Arabidopsis* U02069, *Anemia* X69188, *Chlamydomonas* U31545, *Plasmodium* X62393, *Euplotes* X71353, *Homo* M61764, *Xenopus* M63446, *Drosophila* M61765, *Ustilago* X68132, *Neurospora* X97753, *Aspergillus* X15479, *Schizosaccharomyces* X62031, *Trypanosoma* Y07591, *Reticulomyxa* X97250, *Dictyostelium* AJ000492.

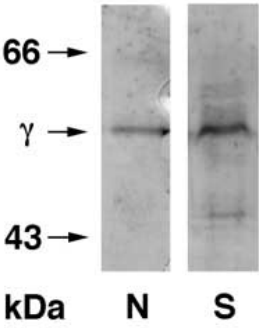

Fig. 4. Immunoblot of nuclear (N) and supernatant (S) fractions of *Dictyostelium* amoebae probed with γ-tubulin antibodies. More than half of the γ-tubulin is present in the supernatant. The positions of molecular mass standards are indicated by arrows.

1995), which shows only 38% identity to *D. discoideum*. An unrooted phylogenetic tree of 15 representative γ-tubulins (excluding *S. cerevisiae* TUB4p) places *D. discoideum* close to the hypothetical origin and in a distant phylogenetic relationship with two protozoan species, *Reticulomyxa* and *Trypanosoma* (Fig. 3).

Immunolocalization

To study the distribution of γ-tubulin in more detail, polyclonal antibodies were produced against a fusion protein of *D. discoideum* γ-tubulin with maltose-binding protein expressed in *E. coli*. Antibodies affinity-purified against γ-tubulin derived from the fusion protein by enzymatic cleavage recognized a 50 kDa polypeptide in bacterial extracts (not shown). In *D. discoideum* slightly more than half of the γ-tubulin is present in the cytoplasm, while the rest is found associated with the centrosome which cosediments with the nuclei due to its tight nuclear association (Fig. 4). To determine the localization of γ-tubulin in *D. discoideum* amoebae, cells were double-labeled with affinity-purified γ-tubulin antibody and a monoclonal antibody against a bona fide centrosomal component of *D. discoideum* (Kalt and Schliwa, 1996). Both antibodies co-localized in a single dot next to the nucleus in interphase cells (Fig. 5). In mitotic cells, γ-tubulin was found in an elongated structure at the spindle poles (Fig. 6). This appearance is consistent with the structure of the spindle pole as seen by electron microscopy, a flattened disc or plaque (Roos and Camenzind, 1981; McIntosh et al., 1985). In addition, the central spindle was also stained.

We were particularly interested to determine the centrosomal distribution of γ-tubulin at the electron microscopic level to take advantage of the more detailed structural information afforded by this technique. The *D. discoideum* centrosome consists of an electron-dense core in which three major layers are discernible, and a less dense corona organized into an array of regularly spaced condensations, or nodules, embedded in a more open, amorphous matrix (Fig. 7a). The maximum number of nodules in the centrosome corona was estimated to be about 60 per interphase centrosome, based on the minimum spacing of about 65 nm of the nodules and the avarage dimensions of a centrosome (280×250×130 nm; see also Moens, 1976). Interphase microtubules were embedded with one end in the corona and, in fortuitous sections, appear to be associated with the nodules. In agreement with earlier studies on *D. discoideum* and related species (e.g. Moens, 1976; Roos, 1975), we found that microtubules may extend at various and sometimes extreme angles from the surface of the centrosome (Fig. 7a,c).

Previous experiments using pre-embedding procedures for immunolocalization at the electron-microscope level with antibodies against γ-tubulin as well as other centrosomal antigens (not shown) revealed localization at the periphery of the corona only, suggesting problems with the access of gold particles to core structures. Therefore, post-embedding

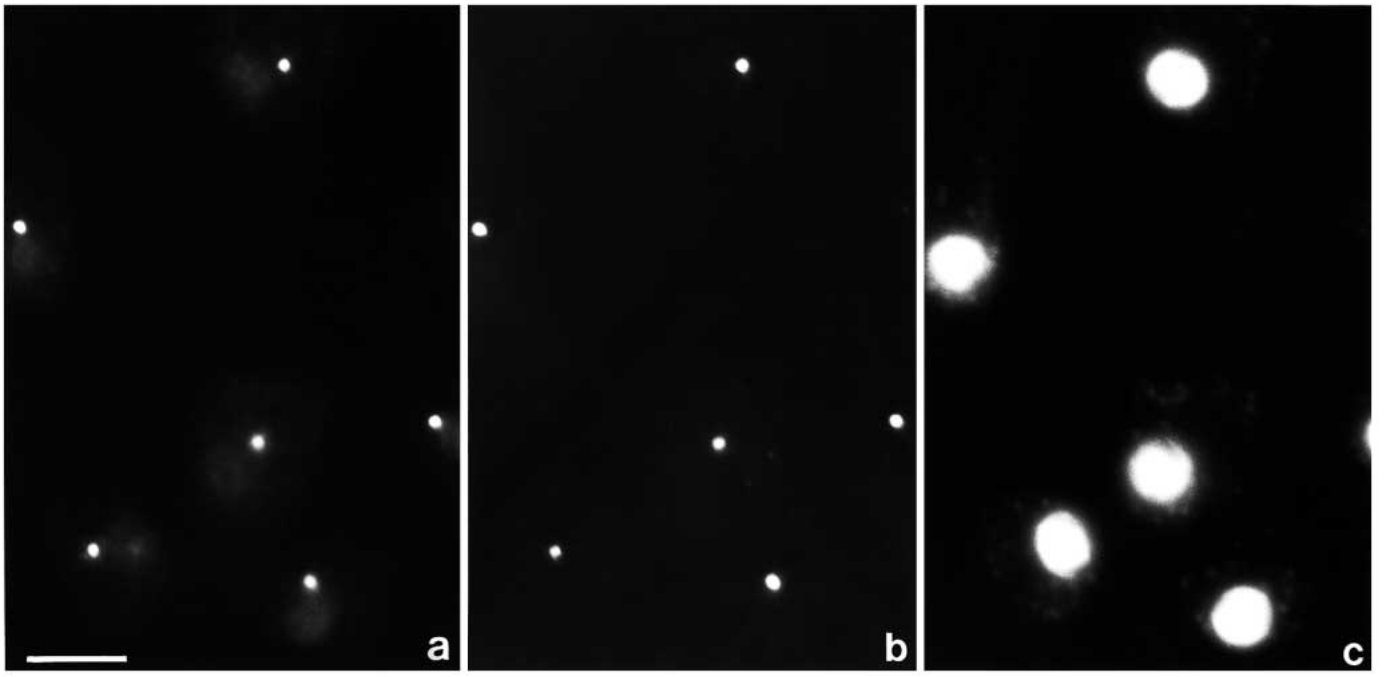

Fig. 5. Immunofluorescence microscopy of interphase AX2 cells stained with antibodies against γ-tubulin (a) and a 350 kDa centrosomal antigen (b). DAPI fluorescence is shown in c. Bar, 5 μm.

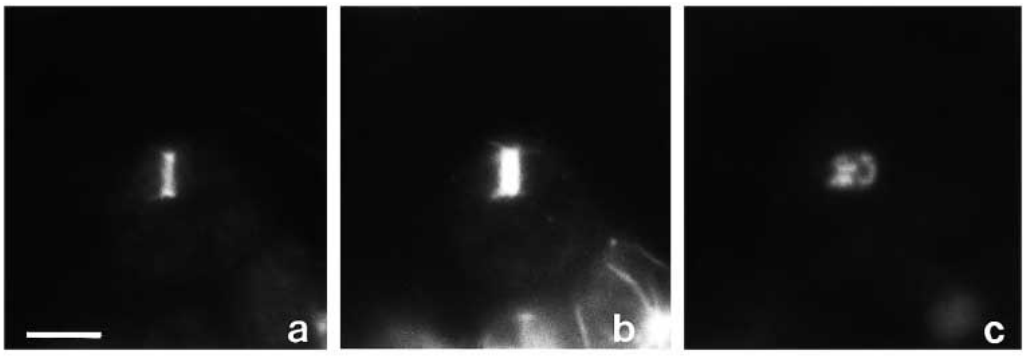

Fig. 6. Immunofluorescence microscopy of a metaphase AX2 cell stained with antibodies against γ-tubulin (a) and α-tubulin (b). DAPI staining is shown in c. γ-Tubulin antibodies label the spindle poles and, somewhat more weakly, the spindle. Bar, 5 μm.

immunolocalization of Lowicryl-embedded cells was employed. Transverse sections of interphase centrosomes showed gold particles to be localized to the central portion of the corona (Fig. 7b,c). Conspicuously, the gold particles are often clustered in groups of 3-8 separated by a space of about 50-60 nm, a phenomenon not seen with other antibodies. The distribution and spacing of these clusters is reminiscent of the distribution and spacing of the nodules within the centrosome corona. In mitotic cells γ-tubulin is associated exclusively with the nuclear face of the centrosome (Fig. 8).

In cells expressing a GFP-γ-tubulin construct the level of γ-tubulin is increased approx. 5-fold (Ueda et al., 1997). Microtubule organization and centrosome function in interphase and mitosis are indistinguishable from control cells, as is the localization of the centrosome-associated GFP-γ-tubulin (Fig. 9; compare with Fig. 7b,c). Thus cells are apparently able to tolerate a modest level of overexpresseion of γ-tubulin without significant deleterious effects.

DISCUSSION

This study provides a molecular characterization of *D. discoideum* γ-tubulin and an analysis of its association with the centrosome. We present a detailed ultrastructural localization of γ-tubulin at this acentriolar centrosome that allows us to correlate its distribution in the centrosome with functional aspects of microtubule nucleation.

D. discoideum γ-tubulin displays significant sequence identity (in the range of 70%) to all other known γ-tubulins, except the highly aberrant γ-tubulin-like TUB4p (Sobel and Snyder, 1995; Burns, 1995b; Spang et al., 1996) which is believed to perform γ-tubulin-related functions in budding yeast. A phylogenetic analysis of known γ-tubulin sequences (excluding TUB4p) using the programs CLUSTAL and PHYLIP revealed that *D. discoideum* γ-tubulin is the most divergent yet reported, with a weak relationship to γ-tubulins from protozoan species. All of the signature peptides of γ-

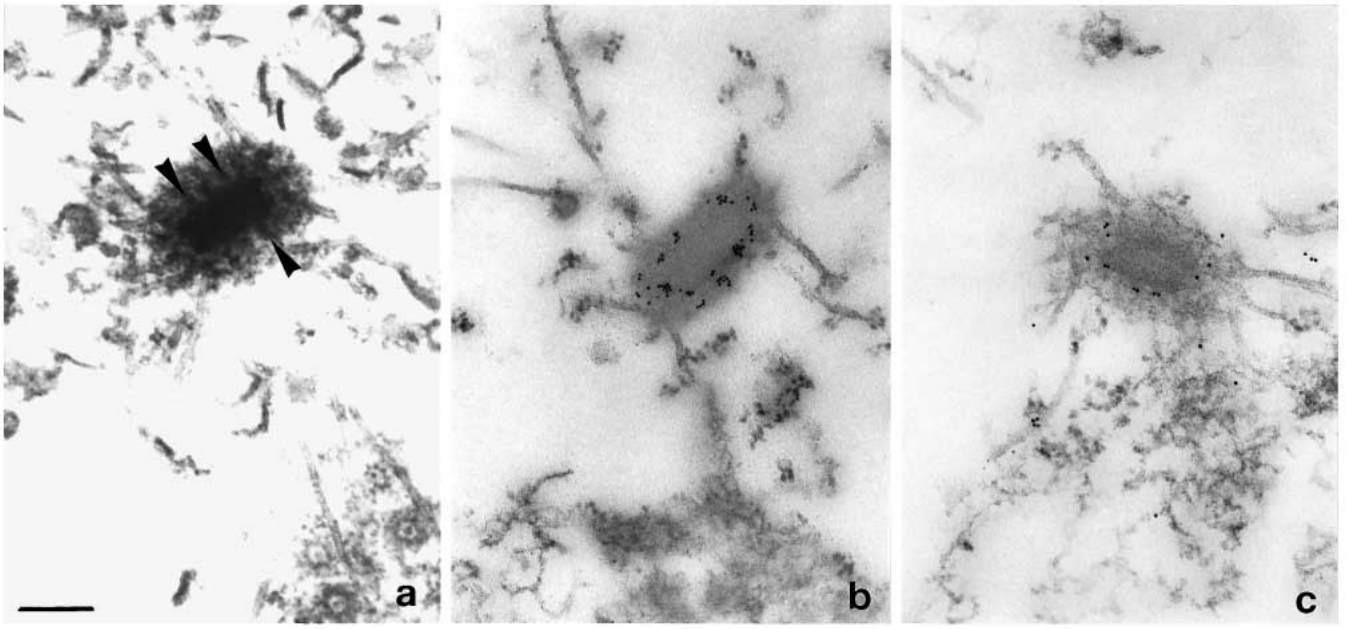

Fig. 7. Electron microscopic localization of γ-tubulin. (a) Centrosome of a cell embedded in epoxy as described in Materials and Methods, showing the electron-dense core surrounded by the corona in which dense nodules of amorphous material are clearly discernible (arrowheads). Several microtubules are seen to radiate from the centrosome. (b) Centrosome of a cell embedded in Lowicryl and labeled with antibodies against γ-tubulin, followed by 6 nm gold-labeled goat anti-rabbit antibodies. Gold particles form clusters within the corona, but not the core. (c) Similar section of a different centrosome labeled with 5 nm Protein-A gold as a secondary marker. Again the gold particles are found in the corona, but more unspecific gold labeling is present. Bar, 0.2 μm (a-c).

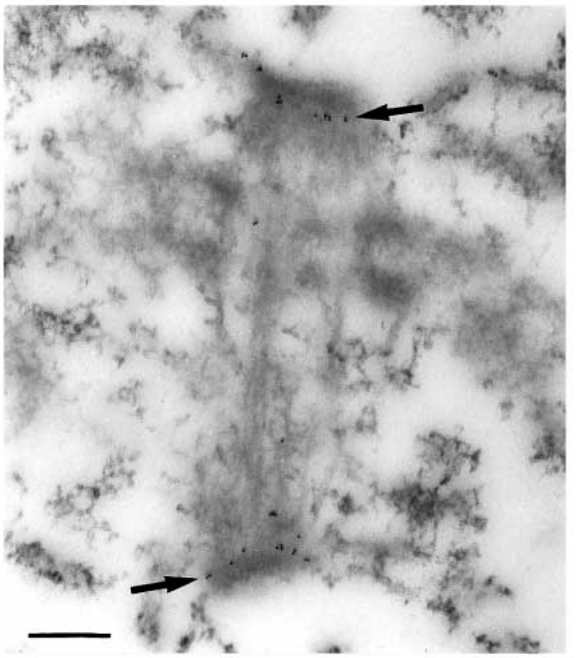

Fig. 8. Localization of γ-tubulin in a Lowicryl-embedded prometaphase cell. Gold particles are found predominantly at the spindle-facing side of the centrosomes (arrows) but also in the spindle region. Bar, 0.2 μm.

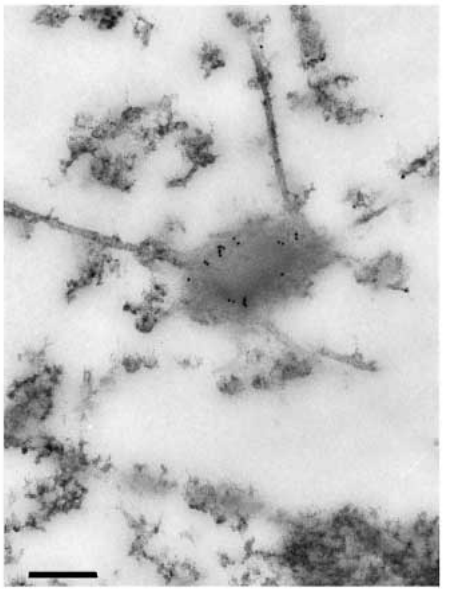

Fig. 9. Localization of GFP-γ-tubulin in a Lowicryl-embedded interphase cell that expresses a GFP-γ-tubulin construct using an antibody against GFP. As in cells reacted with an antibody against γ-tubulin, GFP-γ-tubulin is localized in the corona. Bar, 0.2 μm.

tubulins are present and highly conserved, including the first 6 amino acids at the N terminus which, in a slightly modified form, are implicated in the autoregulation of β-tubulin translation (Yen et al., 1988), and several peptides implicated in the binding of GTP. Interestingly, two of these signature peptides are slightly different from the corresponding peptides of all other γ-tubulins. One, at position 142-148, is SGGTGSG in *D. discoideum*, while it is AGGTGSG in other γ-tubulins. The second is at position 182-184, which is QYN in *D. discoideum* and PYN in all other γ-tubulins. The importance of several other amino acid exchanges that are unique to *D. discoideum* γ-tubulin remains to be determined. Between the amino acid positions 177/178 and 409/410 *D. discoideum* γ-tubulin has deletions of between 2 and 4 amino acids each compared to other γ-tubulins, which presumably result in shortened loop structures in these regions of the molecule. The highly variable C terminus is among the longest of known γ-tubulins and characterized by 8 consecutive asparagines. This is highly unusual for γ-tubulins but not uncommon in other *D. discoideum* proteins which often contain strings of N, I or Q (Kimmel and Firtel, 1985).

D. discoideum possesses a single, compact centrosome from which all cytoplasmic microtubules appear to emerge. This organelle, often referred to as the nucleus-associated body, is linked to the nucleus via as yet unidentified fine filaments (Omura and Fukui, 1985). Our immunofluorescence microscopic studies demonstrate the presence of γ-tubulin at the centrosome in amounts that result in bright fluorescence. In addition, γ-tubulin is also found in crude cytoplasmic extracts as demonstrated by cell fractionation (Fig. 4). This finding is in agreement with observations in other cell types which show that a considerable fraction of γ-tubulin is cytoplasmic (e.g. Felix et al., 1994; Stearns and Kirschner, 1994; Moudjou et al., 1996), presumably as part of a large complex with other proteins that have yet to be identified. Whether the soluble form of *Dictyostelium* γ-tubulin is functionally active in microtubule nucleation cannot be said with certainty, but it appears unlikely since essentially all microtubules of interphase cells are found to be associated with the centrosome, at least on the basis of numerous immunofluorescence microscopic studies. The precise function of non-centrosomal γ-tubulin has, therefore, yet to be determined.

We were particularly interested in the precise localization of γ-tubulin at the centrosome by electron microscopy. Whereas numerous reports have localized γ-tubulin at the light microscopic level using immunostaining, few studies have analyzed the distribution of γ-tubulin at centrosomes by postembedding immunogold electron microscopy in detail. Moritz et al. (1995) used microscopic tomography of isolated *Drosophila* centrosomes to show that γ-tubulin is localized in ring structures within the pericentriolar matrix. These ring complexes apparently are more or less randomly distributed within the pericentriolar material and likely contain several γ-tubulin molecules. Fuller et al. (1995) and Moudjou et al. (1996), in addition to a localization in the pericentriolar matrix, demonstrate the presence of γ-tubulin within the proximal end of the centriolar barrel. Spang et al. (1996) reported that TUB4p, the presumptive γ-tubulin homologue of S. cerevisiae, is localized near the inner and out plaques of isolated spindle pole bodies, structures believed to be involved in microtubule nucleation.

Here we have shown by postembedding immunogold-labeling that γ-tubulin is associated with the *D. discoideum* interphase centrosome in an intriguing pattern: it forms regularly spaced clusters within the corona whose distance from each other correlates well with the spacing of electron-

dense nodules that are a prominent feature of the corona. A direct association of the gold particles with these nodules is difficult to demonstrate because the fixation procedure required for Lowicryl embedding results in reduced contrast compared to conventional fixation and embedding in plastic. γ-Tubulin is not present within the electron-dense, layered core structure of the centrosome, and there is little, if any, γ-tubulin associated with the more open matrix of the corona. This finding suggests an involvement of the electron-dense nodules of the corona in microtubule nucleation. Indeed, single microtubules frequently appear to end (or, rather, begin) at a nodule, and to extend from there into the cytoplasm. These observations lend support to the notion that the corona is the functional homologue of the pericentriolar matrix of 'higher' eukaryotic cells, and that the nodules are the equivalents of the γ-tubulin ring complexes which function as microtubule-nucleating sites in animal centrosomes (Moritz et al., 1995; Zheng et al., 1995). Whether the γ-tubulin in these nodules is part of a ring complex remains to be demonstrated. If one nodule nucleates the growth of one microtubule, for which there is electron microscopic evidence, then the maximum number of microtubules that can be generated by an interphase *D. discoideum* centrosome is about 60 (Kuriyama et al., 1982, and our own calculations). Microtubule counts in cells stained with tubulin antibodies show that, in fact, their number in vivo is lower, around 30. This number, however, may be an underestimate because the ends of short microtubules close to the centrosome may have been missed due to overcrowding with microtubules near the centrosome in immunofluorescence micrographs. Even so, it is reasonable to assume that the number of microtubules nucleated from the centrosome probably does not exceed 40 or 45 in interphase. Thus not all nodules of a given centrosome have associated microtubules at all times. Conceivably, not all nodules are active in microtubule nucleation. Alternatively, the presence of 'empty' nucleation sites may reflect the existence of a steady state of microtubule nucleation at, and release from, the nodules. Studies on cultured epithelial cells demonstrated directly that microtubules nucleated at the centrosome may be released into the cytoplasm where they depolymerize (Keating et al., 1997). Similar events may take place in *D. discoideum*, contributing to a steady turnover of cytoplasmic microtubules.

On the basis of the findings presented here, we propose that γ-tubulin and other proteins required for nucleation are organized into nodules, and that a nodule represents a 'nucleation unit' for a single microtubule. Because the nucleation units are arranged in a fixed spatial pattern on the surface of the centrosomal core, it would make sense to allow for some flexibility of the orientation of the nucleation site itself. Microtubules may be subject to forces that cause them to bend and move in the cytoplasm, leading to the reorientation relative to the site of nucleation and anchorage at the centrosome. In agreement with this view, the angle at which microtubules are seen to extend from the centrosome is quite variable, arguing for a considerable degree of rotational freedom in the microtubule-nucleation unit-linkage.

An intriguing question to be solved in the future is how the molecular components of the nucleation machinery are rearranged when a cell enters mitosis. In contrast to, e.g. a mammalian mitotic centrosome which acquires a much larger pericentriolar matrix but otherwise still resembles the interphase centrosome, the *D. discoideum* centrosome undergoes a shape change and looses the corona. Nodules with a flexible 'hinge' may no longer be needed as spindle microtubules now form an almost crystalline intranuclear spindle (McIntosh et al., 1985). When the transition from the interphase to the mitotic configuration of the centrosome takes place is presently not known, but it presumably occurs during a very short time period. Yumura and Fukui (1987) have evidence that microtubules may be 'shed' from the centrosome prior to spindle formation, and we have found that a prominent structural component of the interphase corona is dispersed near spindle poles in mitotic cells (Kalt and Schliwa, 1996). In late telophase the nodules reappear (Moens, 1976) and the centrosome regains its interphase shape and morphology. These observations demonstrate major transformations in centrosomal morphology, composition, and function upon entry into, and exit from, mitosis in *D. discoideum* that have no equivalent in the well-studied centrosomes of mammalian cells or the spindle pole bodies of yeast. Studies are under way to elucidate these structural transformations and to correlate them with centrosome function.

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