Protoplasma (1998) 203: 118–123

PROTOPLASMA

γ-Tubulin is a component of the Spitzenkörper and centrosomes in hyphal-tip cells of *Allomyces macrogynus*

Rapid Communication

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Received April 15, 1998
Accepted April 29, 1998

Summary. A monoclonal antibody was used to localize γ-tubulin in hyphal tip cells of the chytridiomycete fungus *Allomyces macrogynus*, and its distribution determined with standard epifluorescence and laser scanning confocal microscopy. The results demonstrate that γ-tubulin is a component of the Spitzenkörper and centrosomes. Immunoblot analysis of total soluble protein extracts separated by one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis identified a single 56 kDa γ-tubulin-related polypeptide. Localization of γ-tubulin to the Spitzenkörper of *A. macrogynus* provides evidence that the Spitzenkörper in this fungus functions as a microtubule-organizing center.

Keywords: Immunofluorescence; Microtubule-organizing center; γ-Tubulin; Spitzenkörper.

Abbreviations: BSA bovine serum albumin; DAPI 4′,6-diamidino-2-phenylindole; DIC differential interference contrast; LSCM laser scanning confocal microscopy; MTOCs microtubule-organizing centers; PBS phosphate-buffered saline; SDS-PAGE sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SPB spindle pole body; YpSs yeast extract–inorganic phosphate–soluble starch.

Introduction

Ever since the Spitzenkörper was first reported in the apices of fungal hyphae (Brunswick 1924) it has remained an object of mystery. Little was learned about the nature of the Spitzenkörper until Girbardt (1957) noted the relationships between hyphal growth and its position and presence in the cell. Further investigations of these relationships (e.g., Bartnicki-Garcia et al. 1995, López-Franco et al. 1996) and studies revealing the complexities and variations of Spitzenkörper architecture (e.g., Grove and Bracker 1970, Howard 1981, Vargas et al. 1993, López-Franco and Bracker 1996) have provided a better understanding of the Spitzenkörper and its significance to hyphal tip growth. However, its biochemical composition and the specific function(s) it performs in promoting apical growth remain poorly understood.

In the present study, we have used indirect immunofluorescence microscopy to demonstrate for the first time that γ-tubulin is associated with the Spitzenkörper and centrosomes in hyphal cells of a fungus. Since its identification and characterization in *Aspergillus nidulans* (C. Oakley and Oakley 1989), γ-tubulin has been found in a wide range of eukaryotic cells where it is associated primarily with microtubule-organizing centers (MTOCs) (B. Oakley et al. 1990, Horio et al. 1991, Stearns et al. 1991, Zheng et al. 1991, Muresan et al. 1993, Liu et al. 1993). The localization of γ-tubulin to the Spitzenkörper of *Allomyces macrogynus* implies that the Spitzenkörper in this fungus functions as an MTOC.

Material and methods

Organism and culture conditions

Allomyces macrogynus (Emerson) Emerson and Wilson, strain Burma 3-35, was used throughout this study. Stock cultures were main-

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tained on yeast extract–inorganic phosphate–soluble starch (YpSs) agar (Emerson 1941). Monolayers of growing hyphal tips were prepared for light microscopy as described by Vargas et al. (1993). For protein extraction, flasks containing YpSs broth were inoculated with freshly released zoospores and incubated at 23 °C on a rotary shaker table at 125 rpm. Hyphal colonies were collected for protein extraction after 24 h.

Antibodies

Monoclonal antibodies were used to localize γ-tubulin (IgG; Sigma Chemical Co., St. Louis, Mo.; T-6557) and α-tubulin (IgG; Amersham Corp., Arlington Heights, Ill.; no. 356) for light microscopy and immunoblot analysis. Secondary antibodies included: alkaline phosphatase-conjugated anti-mouse IgG (Sigma; A-5153), bodipy-conjugated antimouse IgG (H + L chain specific; Molecular Probes, Inc., Eugene, Oreg.; B-2752), and Texas red-conjugated anti-mouse IgG (H + L chain specific; Molecular Probes; T-862).

Protein extraction, electrophoresis, and immunoblot

Cells were collected and total proteins extracted according to Roberson and Vargas (1994). Proteins were separated in one dimension on a Protean Cell II (Bio-Rad Laboratories, Richmond, Calif.) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970). Molecular weight standards (Bio-Rad) were run with each gel. After electrophoresis, the proteins were transferred to nitrocellulose membranes using a Trans-Blot semi-dry apparatus (Bio-Rad) according to manufacturers recommendations. Detection of α- and γ-tubulin on nitrocellulose was performed according to methods described by Roberson (1992). Controls included use of an unrelated monoclonal primary antibody (anti-xyloglucan, CCRC-M1; a gift from Dr. Michael Hahn, Complex Carbohydrate Research Center, University of Georgia, Athens, Ga.) and the omission of the primary antibody.

Immunofluorescence staining

Cells were fixed for immunofluorescence microscopy by cryofixation and freeze substitution methods (Roberson and Vargas 1994). Cells were cryofixed by plunging into liquid-nitrogen-cooled liquid propane. Cryofixed hyphae were freeze-substituted for 48 h in cold (–85 °C) methanol containing 1% formaldehyde. Samples were warmed to room temperature, rinsed three times in absolute methanol, rehydrated in a descending series of methanol to deionized water, and transferred to phosphate-buffered saline (PBS). Fixed cells were incubated 8–12 h in the γ-tubulin antibody at 1 : 500 dilution in PBS with bovine serum albumin (BSA) (0.1 M K^+ phosphate, 0.15 M NaCl, 0.1% BSA, 0.02% Na azide, pH 6.8). Following primary antibody incubation, hyphae were rinsed in PBS and incubated for 60 min in Texas red-conjugated anti-mouse secondary antibody diluted 100-fold in PBS with BSA. For colocalization of γ- and α-tubulin, cells were incubated in γ-tubulin antibody followed by a prolonged incubation (8–12 h) in the anti-mouse secondary antibody. The extended incubation time of the secondary antibody ensured the saturation of binding sites on the primary antibody. Cells were rinsed and incubated for 8–12 h in anti-α-tubulin antibody diluted 1 : 500 in PBS with BSA. After rinsing, cells were incubated for 60 min in an anti-mouse secondary antibody conjugated to bodipy-diluted 100-fold in PBS with BSA. Hyphae were rinsed and stained for 15 min with the DNA fluorochrome 4′,6-diamidino-2-phenylindole (DAPI; Sigma) at a concentration of 0.05 μg/ml in PBS. Cells were mounted in 90% glycerol : 10% PBS (0.1 M, pH 8.6) containing 0.1% n-propyl gallate (Sigma) to retard photobleaching. All steps involved in immunofluorescent staining were performed at room temperature. Controls for γ- and α-tubulin labeling included the substitution of an unrelated primary monoclonal antibody (anti-xyloglucan, see above), and the omission of the primary antibody. Because both primary antibodies used in colocalization procedures were monoclonal, the degree to which the first primary antibody (anti-γ-tubulin) was saturated by the Texas red-conjugated anti-mouse antibody was evaluated by omitting the second primary antibody (anti-α-tubulin) and incubating cells directly in the bodipy-conjugated anti-mouse antibody.

Microscopy

Most specimens were examined and images collected with a Leica TCS NT (Leica Imaging System, Exton, Pa.) laser scanning confocal microscope (LSCM) with a Planapo ×100, 1.4 N.A. objective (Figs. 3 and 5). Lasers used for excitation of fluorochromes included an argon/UV laser for DAPI, an argon laser for Texas red, and a krypton laser for bodipy. Appropriate filters were used to visualize each fluorochrome.

Methods used for differential interference contrast (DIC) (Figs. 2 and 4 a) and standard epifluorescence (Fig. 4 b) microscopy and photography have been described previously (Roberson and Vargas 1994, Lowry and Roberson 1997). Micrographs were digitized with a ScanJet IIc (Hewlett Packard, Palo Alto, Calif.).

Digitized images were processed in Photoshop 3.0 (Adobe Systems, Inc., Mountain View, Calif.) on a Macintosh 7100/80AV Power PC (Apple Computer, Inc., Cupertino, Calif.) and printed on an NP-1600M Medical Color Printer (Codonics, Inc., Middleburg Heights, Ohio).

Results

The total proteins from hyphal homogenates of *A. macrogynus* were separated in one dimension by SDS-PAGE and probed with the γ- and α-tubulin monoclonal antibodies by immunoblot methods. The γ-tubulin antibody reacted with a single 56 kDa protein band (Fig. 1, lane A). Reacting hyphal extracts with the α-tubulin antibody resulted in the detection of a major 52 kDa polypeptide band and a faint, lower-molecular-mass breakdown product (Fig. 1, lane B). Omissions of the γ-tubulin (Fig. 1, lane C) and α-tubulin (not shown) primary antibodies or substitution with an unrelated primary antibody (not shown) resulted in no detectable polypeptide bands.

The dominant cytoplasmic features observed in growing hyphal tip cells of *A. macrogynus* were an apically positioned Spitzenkörper and numerous nuclei located throughout the subapical cytoplasm (Fig. 2). Indirect immunolocalization of γ-tubulin in cryofixed and freeze substituted hyphae produced consistent results. In the majority of cells examined (n = 150), γ-tubulin was localized in a spherical to slightly oblong region of the apical cytoplasm (Figs. 3 a, d and 4 b).

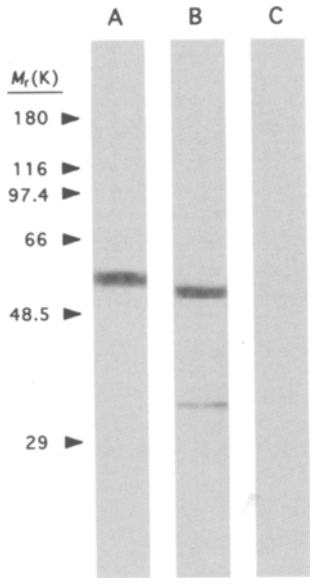

Fig. 1. Immunoblot of *Allomyces macrogynus* hyphal proteins. Positions of molecular-mass markers are noted on the left. *A* Staining of a single γ-tubulin-related polypeptide band (56 kDa) from SDS-PAGE of total proteins. *B* Staining of α-tubulin protein (52 kDa) and lower-mass breakdown product. *C* Omission of the γ-tubulin antibody resulted in no detectable polypeptide bands

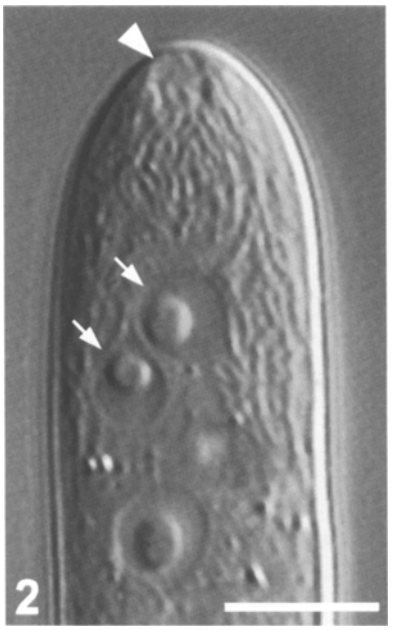

Fig. 2. Growing hyphal tip cell of *Allomyces macrogynus* observed with DIC optics. Median focal plane through hypha reveals an apically positioned Spitzenkörper (arrowhead) and subapical nuclei (arrows). Bar: 5 μm

This region averaged 2.0 μm in diameter (n = 59, S.D. = ± 0.3 μm) and appeared to be uniformly labeled. Colocalization of γ- and α-tubulin showed abundant microtubules (MTs) emanating from the γ-tubulin-rich apical zone (Fig. 3 b, d). In all hyphae, the position of the γ-tubulin zone coincided with that of the Spitzenkörper (Fig. 4), including those hyphae in which the Spitzenkörper had migrated to subapical positions prior to fixation (not shown).

γ-Tubulin was also localized to discrete spots in the subapical cytoplasm (Figs. 3 a, 4 b, and 5). Observations of γ-tubulin and nuclear labeling in the same cell suggested that most of these fluorescent spots were positioned at the periphery of nuclei (Fig. 3 d). Stereo-pair images of similarly labeled cells revealed that two γ-tubulin spots were associated with most nuclei and that these spots were positioned typically at opposite poles of the nucleus (Fig. 5). Spindle MTs were occasionally seen emanating from the γ-tubulin spots (not shown). In approximately half of the hyphae examined by LSCM (n = 20), one to two γ-tubulin spots per cell were not associated with nuclei (Fig. 5).

Omission of primary antibodies or substitution with the anti-xyloglucan antibody produced only slight background fluorescence. In colocalization procedures, elimination of the second primary antibody (anti-α-tubulin) resulted in no detectable labeling of γ-tubulin by the bodipy-conjugated anti-mouse antibody.

Discussion

We have used a monoclonal antibody and indirect immunofluorescence microscopy methods to visualize the distribution of γ-tubulin in hyphal tip cells of *A. macrogynus*. These results demonstrated that γ-tubulin was localized to a spherical region of the hyphal apex and to small spots that were associated generally with nuclei. The specificity of this antibody was demonstrated with immunoblot analysis. Total soluble proteins of *A. macrogynus* contained a single polypeptide (56 kDa) that was immunologically related to γ-tubulin. This band was slightly heavier than γ-tubulin reported in other fungi (50 kDa) (B. Oakley et al. 1990, Stearns et al. 1991), but within the range of molecular masses reported in mammalian (46 kDa) (Gueth-Hallonet et al. 1993, Moudjou et al. 1996) and plant (58 kDa) (Liu et al. 1993) cells.

The apical position and shape of γ-tubulin-related fluorescence correlated with that of the Spitzenkörper as viewed by DIC optics. In hyphae where the Spitzenkörper had migrated to a subapical position

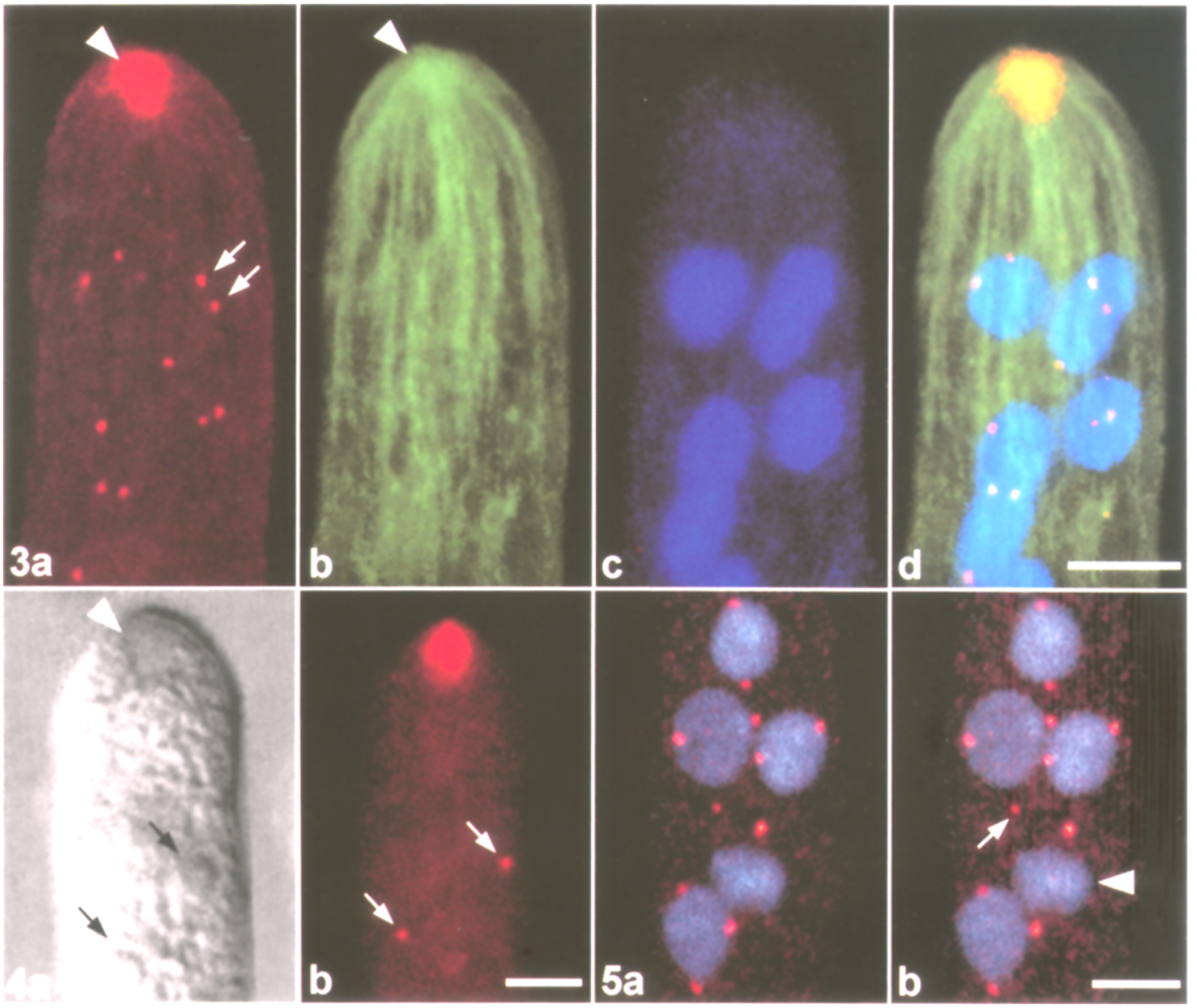

Fig. 3 a–d. A single hyphal tip cell triple labeled for γ-tubulin, α-tubulin, and DAPI viewed with LSCM. **a** γ-Tubulin is shown localized to a spherical region of apical cytoplasm (arrowhead). Subapical γ-tubulin spots (arrows) depict centrosomal labeling. **b** α-Tubulin labeling reveals abundant microtubules emanating from a region located within the hyphal apex (arrowhead). **c** DAPI staining demonstrates the many nuclei located in the subapical cytoplasm. **d** Merged image of panels **a–c**. Note the associations of microtubules with the apical zone of γ-tubulin and the nuclei with centrosomes

Fig. 4 a, b. Hyphal-tip cell prepared for γ-tubulin immunofluorescence. **a** An apically positioned Spitzenkörper (arrowhead) and subapical nuclei (arrows) are visible with DIC optics. **b** γ-Tubulin localization viewed with standard epifluorescence microscopy shows that the apical zone of γ-tubulin corresponds to the position of the Spitzenkörper. Also noted is the association of the centrosomes (arrows) with nuclei (**a**; arrows). **a** and **b** were captured at the same focal plane

Fig. 5 a, b. 6° stereo-pair images of subapical hyphal region labeled for γ-tubulin and nuclei. Images were generated by LSCM. γ-Tubulin is localized to a pair of widely separated centrosomes (red spots) associated with each nucleus. Condensed chromatin (**b**; arrowhead) illustrates the metaphase plate with a single centrosome located above and below. Occasionally, solitary γ-tubulin spots (**b**; arrow) are observed which are not associated with nuclei

prior to fixation, the region of fluorescence remained coincident with the position of the Spitzenkörper. These results lead us to conclude that the Spitzenkörper of *A. macrogynus* contains γ-tubulin. Bourett and Howard (1991) have shown that actin is a component of the Spitzenkörper in *Magnaporthe grisea*. While this cytoskeletal protein is known to be integral to many cellular processes, including maintenance of cell shape and polarity and intracellular transport, its localization per se does not imply a specific function.

In contrast, the association of γ-tubulin with the Spitzenkörper in *A. macrogynus* demonstrates its role as an MTOC.

Previous immunofluorescence and electron microscopy investigations of *A. macrogynus* support the premise that the Spitzenkörper in this fungus is an MTOC (Vargas et al. 1993, Roberson and Vargas 1994). Though the presence of apical MTOCs has been suggested in germ tubes of *Uromyces appendiculatus* (Hoch and Staples 1985), developing asci of *Sordaria macrospora* (Thompson-Coffe and Zickler 1992), and buds of *Saccharomyces cerevisiae* (Snyder 1989), a distinct organelle or cytoplasmic region associated with MTOC function could not be identified clearly.

γ-Tubulin also localized to discrete spots in the subapical cytoplasm of *A. macrogynus*. The association of γ-tubulin spots with nuclear surfaces and mitotic spindle poles suggests they represent centrosomes. This was expected since γ-tubulin has been localized to centrosomes in amphibian, insect, and human cells (Stearns et al. 1991, Zheng et al. 1991, Moudjou et al. 1996) and to spindle pole bodies in fungi (Oakley et al. 1990, Horio et al. 1991). Centrosomes in undifferentiated hyphae of *Allomyces* spp. consist of a single centriole and associated peri-centriolar material (Renaud and Swift 1964, McDaniel and Roberson unpubl. obs.). γ-Tubulin has been localized to basal bodies and centrioles in a variety of cells (Dibbayawan et al. 1995, Fuller et al. 1995, Liang et al. 1996). Immunoelectron microscopy will be required to determine the localization of γ-tubulin within the centrosome of *A. macrogynus*. Interestingly, two widely separated centrosomes were associated with most interphase nuclei in *A. macrogynus* hyphae. It seems that in this fungus centrosomal duplication and migration to opposite poles occurs during late mitosis or early interphase.

The results presented herein contribute to a better understanding of the biochemical and functional characteristics of the Spitzenkörper in *A. macrogynus*. That the *Allomyces* Spitzenkörper regulates MT nucleation, polarity, and distribution, demonstrates its relevance in regulating intracellular motility and maintaining cytoplasmic organization during hyphal tip growth. It is not known whether the Spitzenkörper of other fungi function as MTOCs.

Acknowledgements

Laser scanning confocal microscopy was performed in the W. M. Keck Bioimaging Laboratory (ASU). This work was supported by a grant from the Mycological Society of America (Backus Award; DPM).

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Verleger: Springer-Verlag KG, Sachsenplatz 4–6, A-1201 Wien. — Herausgeber: Prof. B. E. S. Gunning, Research School of Biological Sciences, The Australian National University, Canberra City, ACT 2601, Australien. — Redaktion: Sachsenplatz 4–6, A-1201 Wien. — Hersteller: Adolf Holzhausens Nachfolger, Kandlgasse 19–21, A-1070 Wien. — Verlagsort: Wien. — Herstellungsort: Wien. — Printed in Austria