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Reference


DOI : 10.1111/j.1574-6968.1993.tb05996.x
Evolution of tubulin isoforms during the sporophytic development of *Allomyces arbuscula*

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(Received 7 October 1992; accepted 10 November 1992)

Abstract: Tubulins extracted from the sporophytic developmental stages of *Allomyces arbuscula* have been characterised by one- and two-dimensional SDS-PAGE gels immunoblotted with monoclonal antibodies as α-, acetylated α- (Mr 57 kDa both) and β- (Mr 55 kDa) isoforms. The zoosporangial isoforms could be characterised only when precautions were taken to inhibit the strong tubulin proteolytic activity at this stage. The zoospores and zoosporangia contained greater amounts of the acetylated α- and β-isoforms than the mycelium, while the non acetylated α-isoform was present in greater quantity in the mycelium than in the zoospores or zoosporangia.

Key words: Tubulin; Isoform; Development; *Allomyces*

Introduction

Microtubules have been observed in many fungi [1] such as in the aquatic moulds of the genus *Allomyces* in which they were found to be present in the subapical zone of the vegetative hyphae [2] and in the zoospores [3]. They are known to be involved in flagellar motility [4–6], chromosome segregation [7], maintenance of cellular morphology [8] and intracellular transport of organelles including mitochondria and vesicles [9]. To perform such a variety of functions simultaneously, microtubules must be differentiated either by differential binding of various MAPs, by incorporation of different tubulin isoforms or by post-translational modification of tubulin [10].

The α-, acetylatedα- and β-isoforms of tubulin have already been chemically characterised in the zoospores of *A. macrognynus* [2] and we have detected the presence of the acetylated isoform of α-tubulin in the hyphae of *A. arbuscula* [11]. In this report, we have searched for the tubulin isoforms not only in the vegetative mycelium of the sporophytic developmental phase of *A. arbuscula* (stage 1) but also in differentiating zoosporangia (stage 2) and in the zoospores (stage 3) liberated from mature zoosporangia.
Materials and Methods

Organism and growth conditions
The strain Bali of *Allomyces arbuscula* Buttl. (gift from Dr. C. Stumm) was grown and maintained at 32°C on YpSs medium [13]. To obtain vegetative mycelium, a 40 ml suspension of zoospores was inoculated into 1 l of GCY medium [14] in an Erlenmeyer flask and incubated at 32°C with aeration. After 24 h, the mycelium was harvested, washed with distilled water, dried by squeezing between two blotter papers and stored at −80°C.

Zoosporangia were obtained by inoculating YpSs agar medium with 2–3 ml of a suspension of motile zoospores. These zoosporangia, remaining in their latency stage so long as they were not flooded with a dilute solution of salts, were gently scraped from the surface and stored at −80°C. Zoospores were released within 2 h from zoosporangia differentiated at the tip of hyphae and flooded with 10–20 ml of a dilute solution of salts. They were harvested by gentle cold-pelleting at 1000 rpm for 10 min and stored at −80°C.

Crude extracts

Buffer extraction. Materials from each of the three developmental stages were disrupted in a mortar with liquid nitrogen and quartz sand and then resuspended in a buffer solution (100 mM PIPES, pH 6.9, 1 mM MgSO₄, 1 mM CaCl₂, 2 mM DTT, 0.1 mM GTP, 0.5% Triton X-100) supplemented with protease inhibitors (0.25 μg pepstatin, 10 mM benzamidine, 10 μg/ml leupeptin and 1 mM PMSF) to minimize proteolysis due to endogenous protease activity. Homogenates were centrifuged at 15000 rpm for 15 min and the supernatants were stored at −20°C.

TCA-acetone extraction. Zoosporangia were also disrupted in a mortar with liquid nitrogen and then resuspended in 10% TCA, 0.07% 2-mercaptopethanol in cold acetone according to ref. [12] and kept at −18°C for 1 h. After 15 min centrifugation at 18000 rpm, the supernatant was removed and the pellet was rinsed twice for 30 min at −18°C with 10 ml of cold acetone containing 0.07% 2-mercaptopethanol. The rinsing solution was then carefully removed and the pellet vacuum-dried for 1 h. It was then resuspended in 3 × sample buffer [15], boiled for 5–10 min and stored at −20°C.

One- and two-dimensional gel electrophoresis
Fifty μg of total protein were analysed on 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) according to Laemmli [15] in parallel with the following standard molecular mass markers: phosphorylase b, 94 000; albumin, 67 000; ovalbumin, 43 000; carbonic anhydrase, 30 000; trypsin inhibitor, 20 100 (Pharmacia), and with bovine brain tubulin partially purified in our laboratory according to Shelanski et al. [16].

Two-dimensional-PAGE separation of tubulin isoforms was made by isoelectric focusing [17] using various ampholytes (1/2 pH 3–10; 1/4 pH 4–6; 1/4 pH 5–7; Bio-Rad, Laboratories, Richmond, CA). The isoelectric point for each isoform was determined as following: one microtube gel was migrated according to O'Farrel [17] and cut into 1-cm fragments. Each fragment was flooded with 1 ml distilled water in a 10-ml tube, gently vortexed, and the pH of the different water aliquots measured after 1 h. The correlation between the different pH measures and the position of the spots allowed the determination of the pI of the proteins separated.

Immunoblotting and gel staining proteins
The proteins were detected by Coomassie blue or silver staining procedures. They were electro transferred [18] from unstained gels to nitrocellulose paper BA 8 (pore size 0.45 μm; Schleicher and Shull, Dassel, Germany) using the transfer buffer described by Burnette [19]. The transferred proteins were stained with 0.05% (w/v) Ponceau S in 3% (w/v) trichloroacetic acid (TCA) to confirm their presence. Non-specific binding sites were blocked with 2% BSA in Tris-buffered saline (TBS) (500 mM NaCl, 100 mM Tris·HCl, pH 7.5) for 1 h at room temperature.

The nitrocellulose paper was incubated for 4 h with the commercial anti-α- and anti-β-tubulin monoclonal antibodies (mAbs) raised in mouse against chicken gizzards (nos. 356 and 357, Amersham International, Amersham, UK) diluted 1:2000 in TBS containing 0.5% BSA. After three
washes in TBS, an incubation with the secondary antibody peroxidase-sheep anti-mouse Ig (Amer- sham) was performed for 1 h at room temperature. Following three washes in TBS, peroxidase activity was revealed with 0.5 mg/ml of 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Fluka) in 100 mM Tris·HCl, pH 7.5, containing 0.3% H₂O₂. The reaction was stopped with distilled water. The nitrocellulose paper was also incubated for 4 h with an antibody recognising the acetylated α-tubulin isoform (6-11, B-1, gift from Dr. G. Piperno, Rockefeller University, NY) [20], diluted 1:100 in TBS, followed by further washes in TBS and incubation with the secondary antibody as above.

Results

Buffer extraction

Coomassie blue staining of one-dimensional SDS-PAGE gel revealed the major proteins of

![Fig. 1. SDS–polyacrylamide one-dimensional gel stained with Coomassie brilliant blue showing co-migration of extracts of hyphae (H), zoosporangia (Zp1, buffer; Zp2, TCA-acetone) and zoospores (Z) with bovine brain tubulin and standard molecular mass markers. The arrow indicates the probable position of the tubulin band.](https://academic.oup.com/femsle/article-abstract/107/1/11/628214)

![Fig. 2. SDS–polyacrylamide one-dimensional gels of the hyphal (H), zoosporangial (Zp1, buffer; Zp2, TCA-acetone), zoosporal (Z) and bovine brain extracts (B) immunoblotted with monoclonal antibodies against α-, β-, and acetylated α-tubulin isoforms. MW, molecular mass markers.](https://academic.oup.com/femsle/article-abstract/107/1/11/628214)
the buffer extracts from mycelium, zoosporangia and zoospores. Co-migration of these buffer extracts with the partially purified bovine brain tubulin of 54 kDa [16] showed the probable position of the tubulin band in the three developmental stages (Fig. 1).

Immunoblotting with the α- and β-tubulin monoclonal antibodies revealed one α- and one β-tubulin isoform (molecular mass 57 and 55 kDa respectively) in the hyphae and in the zoospores (Fig. 2). In addition the antibody recognising the acetylated-α isoform revealed an acetylated α-tubulin (Mr, 57 kDa) in growing hyphae and in zoospores. However, in the same gel, the zoosporangial buffer extract did not show any band corresponding to α-tubulin, β-tubulin or acetylated α-tubulin (Fig. 2).

A comparison of the silver-stained (Fig. 3) and immunorevealed (Fig. 4) two-dimensional gels showed the same three tubulin isoforms with a pI between 5.6 and 6. Two α-tubulins were present and designated α1 and α3 [10], α3 being the more acidic, acetylated isoform [21]. β-Tubulin was also present in both hyphae (Fig. 4B,D) and zoospores (Fig. 4A,C). 50 μg of zoosporal extract migrated in a two-dimensional gel silver-stained showed less total proteins than the same amount (50 μg) of mycelial extract but the three tubulin spots were comparable (Fig. 3).

However, and as in the one-dimensional gel, the antibodies against the three tubulin isoforms did not recognise any spot as tubulin in the two-dimensional gel of zoosporangial buffer extract (data not shown).

**TCA-acetone extraction**

The zoosporangial TCA-acetone extract, comigrated in one-dimensional SDS-PAGE gel with mycelial, zoosporangial and zoospore buffer-extracts, was stained by Coomassie blue (Fig. 1). Incubation with the same tubulin monoclonal antibodies as above revealed the presence of one α-tubulin, one acetylated α-tubulin and one β-tubulin (Fig. 2) with the same molecular masses as those revealed in the hyphal and zoosporal extracts.

**Developmentally-related tubulin changes**

Combination of both extraction methods (see above) allowed to show not only that the same three tubulin isoforms were present at the three developmental stages examined, but that their relative concentrations varied. The α1 isoform was present in higher amounts in the hyphae than in the zoosporangia or zoospores, while the acetylated α3 and β isoforms were in greater quantities in the zoosporangia and zoospores than in

![Fig. 3. Two-dimensional silver-stained gels of the extracts of zoospores (Z) and hyphae (H) co-migrated with bovine brain extract (BB) and standard molecular mass markers (MW).](https://academic.oup.com/femsle/article-abstract/107/1/11/628214)
the hyphae. Moreover, non-mature zoosporangia contained same amounts of the different tubulin isoforms as those observed in the free zoospores (Fig. 2).

Discussion

Three isoforms of tubulin, one α, one acetylated α and one β previously detected in the zoosporangia of *A. macrognus* [2] have now been characterised in each of the three main stages of the sporophytic cycle of *A. arbuscula*. The hyphae contain a higher concentration of the α1, the non acetylated isoform, but less of the α3, the acetylated isoform, than the zoospores and the zoosporangia. By contrast, the zoospores and the zoosporangia seem to contain greater amounts of the acetylated α-tubulin isoform than the hyphae. It has been suggested [1,2] that this isoform is associated with structures which are all rather stable and cross-linked with microtubule-binding organelles such as centrioles, flagellum axonemes and kinetosomes [2,20], this in spite of the fact that post-translational modification may not be a prerequisite for microtubule stability and vice
versa [22]. The greater amount of acetylated microtubules found in the zoospores might therefore be related to their need for a stabilizing isoform to provide a stable structural framework during their motile period. This predominance of the α3 over the α1 isoform might also explain why microtubule zoospores are resistant to depolymerising drugs [23].

To ensure their apical growth, the hyphae require a pool of tubulin isoforms ready to polymerise and depolymerise and thereby allowing the maintenance of longitudinal extension. Consequently, they need a dynamic system of microtubules largely comprised of the non-acetylated isoform of α-tubulin, as now found. However, to maintain their subapical, cylindrical shape, hyphae should rather require stable microtubules consisting of the acetylated α isoform as also detected.

Mature zoosporangia ready to discharge zoospores are expected to contain the same amounts of the tubulin isoforms as those observed in free zoospores. It was more surprising to find that zoosporangia, still in their latence phase preceding cleavage of their internal content into zoospores, already contained the same amounts of the three different tubulin isoforms than free zoospores.

As for our finding that tubulin could only be detected in the zoosporangia extracted with an anti-proteolytic, TCA-acetone solution, this has not only allowed the in vitro characterisation of their isoforms but also suggested that, in vivo, microtubules should be in a dynamic state. A neutral protease has been recently purified from A. arbuscula [24] and massively induced in differentiating zoosporangia [25]. This may be implicated in the necessary remodelling of microtubules from a straight to a curved structure allowing the developmental change from the cylindrical shape of the hyphae to that, widely subspherical, of the zoosporangia.

Acknowledgements

We wish to thank Dr. M. Ojha for his useful advice, and Dr. J. Piperno for his generous gift of the monoclonal antibody. We also thank Dr. A.-R. McDonald for her critical reading of the manuscript and Mrs. F. Grange for typing it. The technical assistance of Mr. H. Schönenberger and the photographic expertise of Mr. A. Portianucha were greatly appreciated.

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