Location of a Single β -Tubulin Gene Product in Both Cytoskeletal and Mitotic-spindle Microtubules in *Physarum polycephalum*

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In the mutant BEN210 of *Physarum polycephalum* several β -tubulins are detectable. β 1-tubulin is unique to the myxamoeba, β 2-tubulin is unique to the plasmodium, and the mutant β 1-210 tubulin encoded by the *benD210* allele is present in both cell types. In order to analyse the subcellular distribution of the β 1-210 polypeptide, we prepared cytoskeletons from myxamoebae and mitotic spindles from plasmodia, and examined the tubulin polypeptide composition of these microtubular organelles by two-dimensional gel electrophoresis and immunoblotting. The results show that the β 1-210 tubulin is present in microtubules of both the cytoskeleton and the intranuclear mitotic spindle. Thus a single β -tubulin gene product can participate in multiple microtubular organelles in distinct cellular compartments.

INTRODUCTION

Microtubules, whose principle components are α - and β -tubulin polypeptides, carry out a wide variety of functions in microbial eukaryotes. They form the major structural component of the mitotic and meiotic spindles, and of the cilia and flagella, organelles which mediate motility in a wide range of lower eukaryote cells. The morphology many cells adopt is a reflection of the intracellular arrangement of microtubules in concert with other cytoskeletal proteins. Additionally, many organelles and vesicles are transported intracellularly along microtubule tracks. Generally, in any one microbial cell, several of these functionally and structurally distinct microtubule types exist.

Multiple α - and β -tubulin isotypes exist within cells. This heterogeneity arises from some combination of multiple genes for α - and β -tubulins, and post-translational modifications of tubulin polypeptides (Cleveland & Sullivan, 1985). Considerable interest has developed in the tubulin composition of individual microtubular organelles, a clear question being whether the tubulin isotype composition determines the function of a microtubule (Fulton & Simpson, 1976). One approach to this problem is to study the tubulin composition of isolated microtubular organelles.

In *Physarum polycephalum*, a small multi-tubulin gene family of at least three unlinked β -tubulin genes is observed (Schedl *et al.*, 1984). The *betA* gene, encoding β 1A tubulin, is expressed specifically in the amoebal phase of the life cycle; the *betB* gene, encoding β 1B, is expressed in both the amoebal and plasmodial phases (Burland *et al.*, 1984; E. Paul, unpublished observations); the *betC* gene encoding β 2 tubulin is expressed specifically in the plasmodial phase (Burland *et al.*, 1988). The β 1A and β 1B tubulins are so similar (Singhofer-Wowra *et al.*, 1986) that they co-migrate on two-dimensional gels, but the *betB*-(*benD210*) mutant allele encodes an electrophoretically altered β 1B-tubulin, β 1B210, that can be resolved from β 1A (Burland *et al.*, 1984). Thus all gene products can be analysed simultaneously on the same two-dimensional gel in *benD210* strains.

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In the cellular myxamoeba, microtubules are deployed in the cytoskeleton, mitotic spindle and centrioles (Roobol *et al.*, 1984*a*). Because of the low mitotic index ($< 2\%$), microtubules that are predominantly of cytoskeletal origin can be prepared from myxamoebal cell populations. In the plasmodium, microtubules are present only in the intranuclear mitotic spindle (Roobol *et al.*, 1984*a*). Intranuclear mitosis occurs synchronously in millions of nuclei within the plasmodium, facilitating isolation of mitotic nuclei in buffers that stabilize spindle microtubules (Roobol *et al.*, 1984*b*). Therefore microtubules exclusively from the mitotic spindle can be analysed from the plasmodium. This report compares the different β -tubulin isotypes contained in microtubules obtained from mitotic-spindle and cytoskeleton preparations, allowing assignment of specific gene products to the distinct organelles.

METHODS

Strains of P. polycephalum. Strains CLd (Cooke & Dee, 1975), CLd-AXE (McCullough *et al.*, 1978), BEN210 (Burland *et al.*, 1984) and BEN210-AXE (Foster *et al.*, 1987) have been described. The suffix -AXE describes myxamoebal strains able to grow axenically in liquid medium. CLd-AXE and BEN210-AXE myxamoebae were used for isolation of myxamoebal cytoskeletons. CLd and BEN210 plasmodia were used for isolation of plasmodial mitotic spindles.

Cultures. Microplasmodia, synchronous macroplasmodia and axenic myxamoebae were cultured as described previously (Roobol *et al.*, 1980, 1984*b*).

Isolation of mitotic nuclei. When synchronous plasmodia reached metaphase they were immediately placed in spindle stabilization buffer (4 M-glycerol, 10 mM-PIPES, 5 mM-EGTA, 10 mM-MgCl₂ and 0.1% (v/v) Triton X-100, pH 6.5) at room temperature and processed as described previously (Roobol *et al.*, 1984*b*).

Preparation of cytoskeletons. Myxamoebae were cultured axenically to a density of 4×10^6 ml⁻¹. A total of 2×10^7 cells were washed twice in 0.1 M-PIPES, 2 mM-EGTA, 1 mM-MgSO₄, 50 μ g leupeptin ml⁻¹, pH 6.9 (PE2M1). The myxamoebal pellet was resuspended in PE2M1 with 0.25% (v/v) Triton X-100 by passing it through a pipette. The cytoskeletons were then washed twice in PE2M1 buffer (modified from Clayton *et al.*, 1982).

Immunofluorescence microscopy. Myxamoebae and plasmodia were prepared for this as described previously (Paul *et al.*, 1987). Microtubules were detected with the anti- β -tubulin monoclonal antibody KMx-1 (Birkett *et al.*, 1985).

Two-dimensional gel electrophoresis. In all cases the nuclear pellet, plasmodial biopsy (20 mg), myxamoebal or cytoskeletal pellet was resuspended in 50 μ l boiling CHES buffer (0.1 M-CHES pH 9.5, 2%, w/v, dithiothreitol and 5%, w/v, SDS) and boiled for 5 min. Samples were cooled and 120 μ l solution A and 60 mg urea were added; conditions for electrophoresis were as described previously (Burland *et al.*, 1984).

Immunoblotting. This was done essentially as described by Towbin *et al.* (1979), except that Tris-buffered saline containing 0.05% Tween 20 was used for all antibody binding reactions and for saturating the binding sites of the nitrocellulose (Hancock & Tsang, 1983), and 4-chloro-1-naphthol was used as the substrate for the horseradish-peroxidase-conjugated second antibody (Dakopatts, Denmark).

The β -tubulin isotypes were identified using the monoclonal antibody KMx-1 (Birkett *et al.*, 1985). This antibody is specific for β -tubulin, and recognises all known *Physarum* β -tubulins (Paul *et al.*, 1987).

RESULTS

Pattern of β -tubulin isotypes in whole cells of wild-type P. polycephalum and of a β -tubulin mutant

We have used a monoclonal antibody to β -tubulin and immunofluorescence microscopy to reveal the microtubule arrays of wild-type and mutant myxamoebae and plasmodia (Fig. 1). There are no significant differences between the interphase or mitotic microtubular arrays of wild-type and mutant, indicating that the possession of the mutant β -tubulin has no deleterious effects on the cell. The mitotic indices of wild-type and mutant myxamoebae were determined from immunofluorescence micrographs made from exponentially growing cells. No significant difference was found between wild-type (0.8%) and mutant (1.0%), indicating that the mutant cells are able to pass through mitosis without significant delay – i.e. the mutant BEN210 appears functionally normal by this criterion also.

Wild-type myxamoebae (Fig. 2*a*) have a single β -tubulin electromorph designated $\beta 1$, which consists of at least two polypeptides encoded by different genes (Burland *et al.*, 1984). The BEN-210 mutant myxamoebae (Fig. 2*b*) have an electrophoretically normal $\beta 1$ ($\beta 1A$) and an altered β -tubulin, designated $\beta 1$ -210. The $\beta 1$ -210 tubulin is encoded by the mutant allele *benD210* of the

Fig. 1. Immunofluorescence images of microtubules: (a), (c) and (e) are the *benD*⁺ wild-type and (b), (d) and (f) are the *benD210* mutant. (a) and (b) show the microtubules of the interphase myxamoebal cytoskeleton; (c) and (d) show the microtubule architecture of the open myxamoebal mitotic spindle, whilst (e) and (f) show the microtubule arrangement of the intranuclear plasmodial mitotic spindle.

betB β -tubulin structural gene (Burland *et al.*, 1984). The β 1-210 polypeptide is also expressed in *benD210* plasmodia, along with the plasmodium-specific β 2-tubulin, while the unaltered myxamoebal β 1-tubulin electrophoretic species observed in *benD210* strains is not expressed in plasmodia (Fig. 3 b, and Burland *et al.*, 1984).

In wild-type strains, demonstration of a single protein electromorph does not establish that the electromorph contains only a single gene product, since different genes could encode electrophoretically similar proteins. However, genetic and biochemical analysis has shown unequivocally that the β 1-210 electromorph is indeed the product of a single gene (Burland *et al.*, 1984). The unique electrophoretic mobility of β 1-210, together with its expression in both myxamoebae and plasmodia, facilitates direct study of the presence of this single tubulin gene product in different cell types and in different microtubular organelles.

Presence of β 1-210 tubulin in cytoskeletons and mitotic spindles

To investigate the involvement of β -tubulins in different microtubular organelles, we prepared cytoskeletons from *benD210* myxamoebae and purified nuclei containing metaphase spindles from *benD210* plasmodia. After subjecting these preparations to two-dimensional gel

Fig. 2

Fig. 3

Fig. 2. Detection of the β -tubulin isotypes of myxamoebae of (a) the wild-type CLD-AXE strain and (b) the BEN210-AXE mutant strain. The wild-type strain exhibits only one $\beta 1$ electromorph, consisting of $\beta 1A$ and $\beta 1B$ (Fig. 2 a), whereas the BEN210 mutant strain (Fig. 2 b) has $\beta 1$ ($\beta 1A$) and the $\beta 1$ -210 electromorphs. The β -tubulin isotypes present in the interphase cytoskeletal microtubules of these myxamoebae are analysed in (c) for the wild-type CLD-AXE strain and in (d) for the mutant BEN210-AXE strain. Panel (d) reveals that the mutant $\beta 1$ -210 gene product is present in the cytoplasmic microtubules of the myxamoebal cytoskeleton.

Fig. 3. Detection of β -tubulin isotypes expressed in plasmodia of (a) wild-type and (b) a *benD210* mutant. The wild-type plasmodium expresses $\beta 1$ and $\beta 2$ isotypes whilst the mutant expresses $\beta 2$ and the $\beta 1$ -210 isotypes. The β -tubulin isotypes present in the microtubules of the intranuclear mitotic spindles from the same plasmodia are analysed in (c) for wild-type and in (d) for the *benD210* mutant. Panel (d) reveals that the $\beta 1$ -210 gene product is present in the microtubules of the intranuclear plasmodial spindle.

electrophoresis, the β -tubulins present were examined by immunoblotting using the anti- β -tubulin monoclonal antibody KMX-1. Analysis of the immunoblots revealed that the $\beta 1$ -210 polypeptide was present in microtubules from both the plasmodial intranuclear mitotic spindle (Fig. 3 d) and the myxamoebal cytoskeleton (Fig. 2 d). Therefore a single β -tubulin gene product, the $\beta 1$ -210 polypeptide, is located in two structurally and functionally different microtubular organelles. Since the different β -tubulins are present in the cytoskeleton and spindle preparations in a stoichiometry similar to that observed in the myxamoebal or plasmodial samples, $\beta 1$ -210 tubulin seems not to be selectively enriched in either of these microtubular organelles.

These results also establish that both of the β -tubulins expressed in myxamoebae are used in the cytoskeleton, and confirm that both β -tubulin isotypes expressed in plasmodia are used in the mitotic spindle (Paul *et al.*, 1987).

DISCUSSION

The wide variety of microtubule structures and the identification of differences in the amino acid sequences of different tubulin proteins in the same organism has led to the speculation that individual tubulin isotypes might preferentially function in different microtubule organelles (Fulton & Simpson, 1976). As a first stage examination of this hypothesis we have analysed the tubulin isotype composition of different microtubule organelles in *Physarum*. The *benD210* mutation allowed us to analyse simultaneously the presence of all three β -tubulin gene products in different microtubular structures. $\beta 1B$, the one β -tubulin gene product expressed in both

myxamoebal and plasmodial phases, is utilized not only in two different organelles – cytoskeleton and spindle – but also in two different cell compartments – cytoplasm and nucleus.

Our data are in accord with results from alternative experimental approaches that indicate that multiple tubulins are used non-selectively in different microtubular organelles. In *Drosophila*, the B2t gene is expressed only in the testes; mutation of the B2t gene results in malfunctioning of all the microtubular organelles located in the testes (Kemphues *et al.*, 1982). Disruption of one of the two α -tubulin genes in yeasts (Adachi *et al.*, 1986; Schatz *et al.*, 1986) or the β -tubulin gene normally used in conidiation in *Aspergillus* (May *et al.*, 1985; Weatherbee *et al.*, 1985) does not prevent assembly of the usual microtubular organelles in these organisms. Transfection of various tubulin genes into cultured vertebrate cells leads to incorporation of the transfected gene product into all observable organelles (Lewis *et al.*, 1987). Similarly, antibodies specific for individual tubulins detect each expressed tubulin isotype in all observable microtubular organelles in cultured vertebrate cells (Lewis *et al.*, 1987; Lopata & Cleveland, 1988).

Our analysis of isolated organelles reveals the stoichiometry of the component tubulins, information not available from the more commonly used techniques of immunofluorescent staining of specific tubulin isotypes or mutational disruption. The stoichiometry of β 1A : β 1B in amoebae and β 1B : β 2 in plasmodia was similar in whole cells and specific organelles; this suggests that the cells do not assemble a particular isotype preferentially into particular organelles, but rather that tubulins are incorporated into microtubules in the same proportions at which they are present in the tubulin pool.

Thus, overwhelming evidence has failed to reveal functional specificity for different tubulin gene products, at least at the level of utilization in distinct microtubular organelles, although it is conceivable (but perhaps unlikely) that differential utilization of individual tubulin isotypes could be observed if one examined individual microtubules. It seems most likely that the tubulin isotype composition of a microtubular organelle is not the determinant that specifies the function of the microtubule. It is plausible that tubulin is simply the main building block of the microtubule and that microtubular function is determined by other mechanisms, such as post-translational modifications (Gard & Kirschner, 1985; Piperno & Fuller, 1985; Raybin & Flavin, 1977) and involvement with microtubule-associated proteins (Parysek *et al.*, 1984).

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