Physarum plasmodia do contain cytoplasmic microtubules!

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Summary

It has been claimed that the plasmodium of the myxomycete Physarum polycephalum constitutes a very unusual syncytium, devoid of cytoplasmic microtubules. In contrast, we have observed a cytoplasmic microtubule network, by both electron microscopy and immunofluorescence in standard synchronous plasmodia, either in semi-thin sections or in smears, and in thin plasmodia, used as a convenient model. Cytoplasmic microtubules could be seen after immunofluorescent staining with three different monospecific monoclonal anti-tubulin antibodies. The immunolabelling was strictly restricted to typical microtubules as shown by electron microscopy. These cytoplasmic microtubules were entirely and reversibly disassembled by cold treatment and by either of two microtubule poisons: methyl benzimidazole carbamate and griseofulvin. The microtubule network, present in all strains that have been studied, contains single microtubules and microtubule bundles composed of two to eight microtubules. Cytoplasmic microtubules form a dense and complex three-dimensional network, distinct from the microfilamentous domains and from the nuclei. The orientation of the microtubule network varies according to the plasmodial domain examined. Generally microtubules show no special orientation except in plasmoidal veins where they are oriented parallel to the long axis of the veins. Differences between our observations and those of previous workers who failed to find cytoplasmic microtubules in plasmodia are discussed. We propose that they reflect difficulties of observation mainly due to the fluorescent background. In contrast with the previous view, the discovery of a microtubule cytoplasmic cytoskeleton in Physarum plasmodia raises several questions concerning its relationships with other cellular organelles and its dynamics during different cell cycle events.

Key words: microtubule, microtubule disassembly, microtubule poisons, griseofulvin, methyl benzimidazole carbamate, myxomycete, Physarum, syncytium.

Introduction

The microtubular and microfilamentous cytoskeletonS have been extensively studied in the plasmodium of Physarum, a large syncytium reaching several cm in diameter and containing up to 10⁹ nuclei. This macroscopic cell is able to move on its substratum and exhibits vigorous cytoplasmic streaming (Sachsemair et al. 1972) in cytoplasmic differentiated veins; these movements involve an actomyosin network (Porter et al. 1965; Kessler, 1982). Microtubules have been observed only in the intranuclear spindle during mitosis (Guttes et al. 1968; Goodman and Ritter, 1969; Ryser, 1970; Sakai and Shigenaga, 1972; Wille and Steffens, 1979; Havercroft and Gull, 1983) and, with the exception of one unconvincing report (McManus and Roth, 1967), it has been repeatedly claimed that plasmodia are devoid of cytoplasmic microtubules (Green et al. 1987; Burland et al. 1983; Paul et al. 1987; Burland et al. 1988; Eon-Gerhardt et al. 1981; Gull and Trinci, 1974; Roobol et al. 1984; Birckett et al. 1985a; Solnica-Krezel et al. 1988; Sauer, 1982; Burland, 1985; Laffler and Tyson, 1985; Anderson et al. 1985; Solnica-Krezel et al. 1990). In this respect Physarum plasmodia seem to constitute a very unusual eukaryotic cell.

Despite these numerous reports, the absence of cytoplasmic microtubules was based upon a few observations by electron microscopy (Dugas and Bath, 1962; Rhea, 1966; Porter et al. 1965; Havercroft and Gull, 1983) and immunofluorescence staining (Havercroft and Gull, 1983; Solnica-Krezel et al. 1990). In contrast, several lines of evidence have suggested that microtubules might be present during interphase. First, although tubulin is rapidly degraded after mitosis (Carrino and Laffler, 1985; Ducommun and Wright, 1989), the amount of tubulin reaches a plateau at the end of S-phase until the next cyclic synthesis of tubulin occurring in late G₂-phase (Carrino and Laffler, 1985; Ducommun and Wright, 1989). Second, the heat-soluble 125 kDa polypeptide, a protein known to bind to microtubules in vitro, is phosphorylated twice during the cell cycle: at late S/early G₂-phase and at late G₂/prophase stage (Albertini et al. 1990). The last phosphorylation event could possibly be correlated with the formation of the mitotic spindle, but the former could not be understood, given the apparent absence of microtubules during interphase. Electron microscopy and immunofluorescence observations have unambiguously demonstrated that the intranuclear spindle is nucleated, during early prophase, on an intranuclear spindle organizing center (Sakai and Shigenaga, 1972; Blessing, 1972; Tanaka, 1973), and disappears in less than two minutes at the end of telophase (Goodman and Ritter, 1969; Guttes et al. 1968). Thus it is likely that any putative interphase microtubules would be located in the cytoplasm.
In this report we demonstrate, using appropriate conditions for both electron microscopy and immunolabeling, that the cytoplasm of Physarum plasmodia contains a three-dimensional network of microtubules that has no obvious relationship with the nuclei.

Materials and methods

Physarum plasmodia

Microplasmodia (strains CL, M3CIV, TU291, CH713×CH957, CH713×LU860) were grown at 22°C in stirred cultures (Daniel and Rusch, 1961; Daniel and Baldwin, 1964) and used to prepare synchronous plasmodia (Guttes and Guttes, 1964). Microplasmodia were fused for 2h on the center of a nitrocellulose membrane in the absence of nutrients. The resulting standard plasmodium, grown on semi-defined agar medium (Wright and Toller, 1978), was synchronous as judged by the observation of mitotic stages (Guttes and Guttes, 1964). Thin plasmodia were obtained from square plasmodial pieces (3 mm × 3 mm) cut, with the supporting membrane, from the growing edge of standard plasmodia during the third cell cycle following microplasmodial fusion. Plasmodial pieces were placed on a thin film (1 mm) of 1% agar spread on a glass slide (Naib-Majani et al., 1983), covered with a cellophane membrane and sandwiched with another sheet of 1% agar (thickness 4 mm). After 6h the specimens reached a diameter of 1–2 cm and were taken at intervals. A smear was observed by phase-contrast microscopy (Guttes and Guttes, 1964), while the remaining part was fixed for immunofluorescence. The number of nuclei in thin plasmodia was determined by immunofluorescence in a Malassez slide: the plasmodia were homogenized in 0.25 M Na2HPO4 (pH 7.2), 0.13 M sodium chloride, 0.1% Triton X-100, and processed for immunofluorescence with the antibodies YL1/2 (Kilmartin et al., 1982) and TPH4. Second, observation of cytoplasmic microtubules was performed on a laser scan microscope (Zeiss LSM 10) equipped with a 488 nm argon laser, used at 10% of its maximal power, and a x63 Plan-apochromat objective (NA 1.4). Images were photographed on a black and white monitor with a Nikon 35 mm camera (macro 50 mm lens) using Kodak T-Max film with an exposure time varying from 0.25 to 0.5 s.

Preparation of the plasmodia for immunofluorescence

Immunofluorescence studies were done with three different types of plasmodial preparations: (a) thin plasmodia with or without detergent extraction; (b) semi-thin sections of standard plasmodia; and (c) smears from standard plasmodia. In all cases methanol was used for fixation, since the use of other fixation methods was found unsatisfactory. First, thin plasmodia were fixed for 15 min in cold methanol (−20°C), kept in 3.5 mM potassium phosphate buffer (pH 7.2), 0.13 M sodium chloride (PBS) and processed for immunofluorescence with the antibodies YL1/2 and KMX 1. Alternatively, thin plasmodia were incubated for 5 min in 60 mM Pipes (piperazine-N,N'-bis [2-ethanesulfonic acid], 25 mM HEPES (N-2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid), 10 mM EGTA (ethyleneglycol-bis(aminooxy)ethane-N,N',N',N'-tetraacetic acid), 2 mM magnesium chloride, pH 6.9 (PHEM), containing 0.25% Triton X-100 and fixed for 15 min in cold methanol before immunofluorescence with the antibody TPH4. Second, observation of cytoplasmic microtubules in semi-thin sections prepared from standard macroplasmodia was done in the following way: plasmodial pieces (0.5 cm × 0.5 cm) were cut, with the supporting filter, from standard synchronous plasmodia between mitosis II and mitosis III (third cell cycle), incubated for 5 min at 22°C in PHEM containing 0.25% Triton X-100 and fixed for 10 min in methanol at −20°C. Then the plasmodial pieces were washed in PBS, embedded in Tissue-Tek (Miles) and frozen in liquid nitrogen. Semi-thin sections (4–6 μm) were obtained, placed on slides covered with 1% gelatin and processed for immunofluorescence.

Immunofluorescence

The rat monoclonal antibody YL1/2 (Kilmartin et al., 1982) was used for all immunofluorescence observations of microtubules unless otherwise stated. Plasmodia were reacted for 1h at 37°C with anti-tubulin antibodies diluted in PBS containing 25% fetal calf serum, then washed twice in PBS containing 0.05% Tween-20 and in PBS. Then plasmodia were reacted with goat anti-rat or goat anti-mouse antibodies labelled with fluorescein, diluted in PBS containing 40% fetal calf serum, for 30 min at 37°C and then washed as before. Nuclei were stained with DAPI (0.2 μg ml−1 in PBS) for 15 min at 37°C and washed twice for 5 min in PBS. Preparations were mounted in Mowiol (Planques et al., 1989) and observed with a confocal laser microscope (Radiance 2000, Bio-Rad Laboratories) or an Axioshot microscope equipped with a 40× Plan-neofluar objective (NA, 0.90), an ×63 Plan-apochromat objective (NA, 1.4) and ×100 Plan-neofluar objective (NA, 1.30), an Optovar varying from x1.25 to x2.5 and a x4 TV camera adaptor. Images, recorded with a Sony camera (model DXC-910) and processed for electron microscopy. Alternatively, images were observed by epifluorescence using a Zeiss Axioshot microscope equipped with a ×40 Plan-neofluor objective (NA, 0.90), an ×63 Plan-apochromat objective (NA, 1.4) and ×100 Plan-neofluor objective (NA, 1.30), an Optovar varying from x1.25 to x2.5 and a x4 TV camera adaptor. Images, recorded with a Sony camera (model DXC-910) and processed for electron microscopy. Alternatively, images were observed by epifluorescence using a Zeiss Axioshot microscope equipped with a ×40 Plan-neofluor objective (NA, 0.90), an ×63 Plan-apochromat objective (NA, 1.4) and ×100 Plan-neofluor objective (NA, 1.30), an Optovar varying from x1.25 to x2.5 and a x4 TV camera adaptor. Images, recorded with a Sony camera (model DXC-910) and processed for electron microscopy. Alternatively, images were observed by epifluorescence using a Zeiss Axioshot microscope equipped with a ×40 Plan-neofluor objective (NA, 0.90), an ×63 Plan-apochromat objective (NA, 1.4) and ×100 Plan-neofluor objective (NA, 1.30), an Optovar varying from x1.25 to x2.5 and a x4 TV camera adaptor. Images, recorded with a Sony camera (model DXC-910) and processed for electron microscopy. Alternatively, images were observed by epifluorescence using a Zeiss Axioshot microscope equipped with a ×40 Plan-neofluor objective (NA, 0.90), an ×63 Plan-apochromat objective (NA, 1.4) and ×100 Plan-neofluor objective (NA, 1.30), an Optovar varying from x1.25 to x2.5 and a x4 TV camera adaptor. Images, recorded with a Sony camera (model DXC-910) and processed for electron microscopy.

Electron microscopy

Plasmodial pieces, cut from standard macroplasmodia, were lysed for 3 min at 22°C in PHEM containing 0.25% Triton X-100, fixed for 3–4h in PHEM containing 2.5% glutaraldehyde and washed twice for 30 min in 100 mM sodium cacodylate, 2 mM magnesium chloride (pH 7.2). The plasmodial pieces were post-fixed for 4h at 0°C in 100 mM sodium cacodylate, 2 mM magnesium chloride, containing 1% osmium tetroxide, washed in the same medium, dehydrated and embedded in Epon-812. Thin sections (100 nm), obtained with a Reichert ultramicrotome G02-2, were stained for 5 min with 5% uranyl acetate and for 3 min with lead citrate. Before staining, some thin sections were pretreated with 1.1 vol. hydrogenated water. Pictures were obtained with a Philips electron microscope EM-301. Thin plasmodia were lysed for 2 min at 22°C in PHEM containing 0.1% Triton X-100 and 10% dimethyl sulfoxide, fixed for 30 min in PHEM containing 10% dimethyl sulfoxide and 2% glutaraldehyde. Then they were washed three times for 10 min in PHEM/dimethyl sulfoxide, treated for 10 min at 22°C in PHEM containing 0.1% Triton X-100 and processed for electron microscopy. Alternatively, plasmodia fixed in the presence of 4% tannic acid were used to count the number of microtubule protofilaments. In order to immunolabel cytoplasmic microtubules, a thin plasmodium was lysed for 2 min at 22°C in PHEM containing 0.25% Triton X-100, fixed for 90 min at 22°C in PHEM containing 2.5% glutaraldehyde and washed in the same medium without glutaraldehyde for 30 min.

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Then it was incubated for 1h in PHEM containing 2.5% ammonium chloride and washed extensively for 30 min in PHEM. The plasmodium was incubated for 1h at 4°C in the presence of the antibody against tubulin (YL 1/2, diluted in 10 mM Tris-HCl), 150 mM sodium chloride, pH 7.0 (TBS), containing 10% fetal calf serum, and successively washed in TBS for 5, 10, 25 and 60 min. Then the plasmodium was incubated for 90 min in the presence of gold-labelled goat anti-rat IgG (Janssen, diluted in TBS containing 25% fetal calf serum) and washed in TBS for 5, 10 and 120 min. After a second fixation for 1 h at 24°C in TBS containing 1% glutaraldehyde, the plasmodium was post-fixed for 30 min in 2% osmium tetroxide and mounted for electron microscopic observation.

Results

Immunofluorescent labelling of microtubules in the cytoplasm of Physarum plasmodia

The microfilamentous cytoskeleton of Physarum plasmodia has been most easily studied by immunofluorescence when thin and flat specimens are used (Kamiya and Kuroda, 1965; Naib-Majani et al. 1982; Naib-Majani et al. 1983). Small plasmodial pieces, on a supporting membrane were cut from large standard synchronous macroplasmodia and sandwiched between two pieces of agar (Naib-Majani et al. 1983). This procedure flattened the plasmodium without interfering with cell cycle events, as shown by the occurrence and completion of mitosis. The agar-sandwich plasmodial preparations gave the clearest images and were therefore used most. However, the other plasmodial preparations obtained directly from standard synchronous plasmodia also revealed microtubular elements by immunofluorescence.

Three different monoclonal anti-tubulin antibodies were used to reveal cytoplasmic microtubules by immuno-fluorescence. Their specificity to tubulin polypeptides was checked by immunoblotting plasmodial extract submitted to one-dimensional polyacrylamide gel electrophoresis (not shown). The two monoclonal antibodies, TPH 4 and KMX 1, recognized only one polypeptide band corresponding to the α and β-tubulin isotypes, respectively. But, in the conditions used, no immunolabelling was observed with the YL 1/2 monoclonal antibody, in contrast with previous observations on Physarum purified tubulin showing that this antibody was specific for tubulin α1 and β1 isotypes terminated by an aromatic amino acid (Green et al. 1987; Burland et al. 1983; Gull et al. 1987). The YL 1/2 monoclonal antibody labelled numerous elongated structures (Fig. 1A) in the cytoplasm of interphase plasmodia (Fig. 1B) forming a dense meshwork without any special orientation. When this antibody was deleted from the immunolabelling protocol only a diffuse background of fluorescence was observed (Fig. 1C). Similar immunolabelling (Fig. 1D) was obtained with the KMX 1 monoclonal antibody, which recognizes β1 and β2-tubulin isotypes (Birkett et al. 1985b) and with the TPH 4 monoclonal antibody specific for the α1-tubulin isotype (Planques et al. 1989) (Fig. 1E and F, respectively). In both cases, no elongated structures were observed when these antibodies were omitted from the immunolabelling protocol (not shown). The fixation procedure used with the YL 1/2 monoclonal antibody could be used also with the KMX 1 monoclonal antibody and in both cases the fluorescent background was very low (Fig. 1D and E). In contrast, a different preparation procedure had to be applied for a successful immunolabelling with the TPH 4 monoclonal antibody and the level of background fluorescence was higher (Fig. 1F) than in the two previous cases. Thus it is likely that the elongated structures, which were immunolabelled with three distinct monoclonal antibodies, corresponded to cytoplasmic microtubules. However, these observations did not permit the distinction between isolated microtubules and small bundles composed of several microtubules parallel to each other.

The presence of cytoplasmic microtubules in thin plasmodia was not an indirect consequence of the flattening treatment. Cytoplasmic microtubules were also observed after immunofluorescent labelling in semi-thin sections (Fig. 1G) and in smears (Fig. 1H) obtained from standard synchronous macroplasmodia during the third synchronous cell cycle. Although plasmodial sections exhibited an apparent thickness of 4–6 μm, which was similar to the thickness of thin plasmodia, the observation of microtubules by immunofluorescence was perturbed by the presence of large regions exhibiting an important fluorescence. The elongated structures that were immunolabelled were generally thicker than those that were observed in thin plasmodia. Moreover, except in some limited areas (Fig. 1G, upper left), they did not form a complex meshwork. It is likely that the high fluorescence background limited the observations to the larger microtubule bundles. In smears, the fluorescence background was reduced and more diffuse (Fig. 1H), but the perturbations induced by the shearing of the plasmodial pieces prevented the study of the spatial organization of the cytoplasmic microtubules.

The failure of previous workers to observe cytoplasmic microtubules is unlikely to be a consequence of the strains used, since we found such microtubules in all plasmodial strains that were tested: CL (Fig. 1F and Fig. 5E–G), M3CIV (Fig. 5H), TU291, CH957×CH713 and LU860×CH713 (not shown).

Sensitivity of plasmodial microtubules to cold treatment and microtubule poisons

Cytoplasmic microtubules were not disassembled by a 1 h treatment at 5°C but were entirely disassembled after 15 min at 0°C (Fig. 2A (control) and Fig. 2B (treatment at 0°C for 15 min)). Microtubule disassembly was reversible. When plasmodia treated for 1 h at 0°C were rewarmed to 27°C the cytoplasmic microtubule network reassembled. As expected from the absence of action of taxol (20 μM) on Physarum mitosis in vivo (Wright et al. 1982), this microtubule poison did not stabilize cytoplasmic microtubules from cold disassembly.

Cytoplasmic microtubules were entirely disassembled by a 30 min treatment with 200 μM methyl benzimidazole carbamate or 200 μM griseofulvin, two microtubule poisons already known to act on plasmodial mitosis (Wright et al. 1976; Gull and Trinci, 1974; Hebert et al. 1980; Burland et al. 1984). No microtubules remained in the cytoplasm after 0.5 h in the presence of 200 μM methyl benzimidazole carbamate (Fig. 2C) or 200 μM griseofulvin (Fig. 2D). The initial disassembly of cytoplasmic microtubules by methyl benzimidazole carbamate and by griseofulvin led to a complete absence of immunolabelling of tubulin assemblies both in the cytoplasm and in the nuclei. However, after 4 h of treatment with 200 μM methyl benzimidazole carbamate most nuclei showed the presence of intra-nuclear tubulin assemblies (Fig. 2E and G), in agreement with the previous observations by electron microscopy (Wright et al. 1976; Gull and Trinci, 1974; Hebert et al. 1980). In contrast, even after 6 h of treatment with 200 μM griseofulvin only a few nuclei showed intra-nuclear tubulin assemblies (Fig. 2F and H). The action of Physarum plasmodia contain cytoplasmic microtubules
Fig. 1. For legend see p. 514
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Fig. 2. For legend see p. 514
griseofulvin was completely reversible. A thin plasmodium treated for 6 h with 200 μM griseofulvin and incubated for 5 h in the absence of the drug showed no microtubulin assemblies in the nuclei and a typical microtubule network in the cytoplasm. In contrast, the action of methyl benzimidazo carbamate was less readily reversible. After a 6 h incubation in the presence of 100–200 μM methyl benzimidazo carbamate, followed by a further 1.5 h incubation in the absence of the drug, microtubules were observed in the cytoplasm. A 6 h incubation in the presence of 50 μM methyl benzimidazo carbamate led to the complete disappearance of the cytoplasmic microtubule network, without inducing the assembly of microtubulin in the nuclei. When this treatment was followed by 1.5 h incubation in the absence of the drug, the cytoplasmic microtubule network reappeared, showing that under these conditions the action of methyl benzimidazo carbamate was fully reversible.

The absence of the cytoplasmic immunofluorescent network after incubation of plasmodia at 0°C or treatment in the presence of methyl benzimidazo carbamate or griseofulvin confirms that the elongated structures observed by immunolabelling with antibodies against tubulin are indeed microtubules. Moreover, the complete disappearance of the cytoplasmic microtubule network suggests that these microtubules do not constitute a heterogeneous class of organelle, in contrast to what has been reported in other cells (Gundersen et al. 1984; Kreis, 1987; Schulze and Kirchner, 1987; Piperno et al. 1987).

Electron microscopic observation of plasmodial cytoplasmic microtubules

Both in standard synchronous plasmodia and thin plasmodia, cytoplasmic microtubules were observed by electron microscopy (Fig. 3). They exhibited typical inner and outer diameters (Fig. 3A, B and C) and 13 protofilaments in cross-section (not shown). Immunogold labelling obtained with the anti-tubulin antibody YL 1/2 demonstrated that gold particles were observed in close association with cytoplasmic microtubules, while a complete absence of gold particles did not coincide with a remaining cytoplasm (Fig. 3D). These observations confirm that the elongated structures observed in the cytoplasm by immunofluorescent labelling corresponded to microtubules.

Using serial thin sections, it has been possible to record single microtubules over 6 μm in length. The difficulties of following microtubules over long distances in thin sections account for the observation of smaller lengths by electron microscopy than by immunofluorescent staining. It was clear from electron microscopic observations that the cytoplasmic microtubules constituted a three-dimensional network. Although, observation of thin sections by electron microscopy did not easily permit the determination of the overall structure of the cytoplasmic microtubule network, it was clear that cytoplasmic microtubules had no preferential orientation relative to the whole plasmodium. In numerous cases microtubules have been observed underlying the plasma membrane. Numerous images suggest that cytoplasmic microtubules can exhibit an overall orientation similar to that of the microfilaments (Fig. 3E). It is also clear that portions of the microtubule network are independent of the microfilamentous cytoskeleton (Fig. 3E).

Immunofluorescence did not permit distinction between single microtubules and bundles composed of a few microtubules (Inoué, 1986). However, electron microscopic observations showed that in more than 50 % of the cases, single microtubules were observed (Fig. 4A, C). Groups of 2–8 microtubules were frequent (Fig. 3A, B and C) and 75 % of assembled tubulin consisted of small microtubule bundles rather than single microtubules (Fig. 3D). Half of the microtubules (58.7 %) were found in bundles of 2–5 microtubules, while bundles of 6–8 microtubules were observed with a frequency about 5 % (Fig. 4). The experimental distribution of the number of microtubules per bundle (Fig. 4A, C) did not correlate with a Poisson distribution (Fig. 4B). Less microtubule bundles contained 2 microtubules and more microtubule bundles contained 4–8 microtubules than would be expected from a Poisson distribution. Thus, it is unlikely that microtubule bundles could result only from the random association of microtubules with one another, suggesting the presence of microtubule nucleating centers in the cytoplasm. The cytoplasmic microfilamentous and microtubule skeletons are quite distinct by their size (Fig. 3E). Although microfilamentous domains occupy large cytoplasmic regions, the complex cytoplasmic microtubule network is composed mainly of single microtubules and...
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Fig. 3. Electron-microscopic observation of cytoplasmic microtubules in standard and thin plasmodia. (A) Transverse section of two microtubule bundles close together in a standard macroplasmodium. One microtubule bundle is composed of two microtubules, while the other is composed of four microtubules. (B and C) Longitudinal section of microtubule bundles in a thin plasmodium (B) and in a standard macroplasmodium (C). (D) Longitudinal section of a thin plasmodium showing a microtubule immunolabelled with the anti-tubulin antibody YL 1/2. The gold particles (10 nm) are strictly limited to the microtubule. (E) Section of a standard macroplasmodium showing both the microtubule and the microfilament networks (*). Although some microtubules are more or less parallel to the microfilaments, other microtubules are either perpendicular to the microfilament network (arrowhead) or show no apparent interactions with the microfilaments (arrow). Bars, 0.2 µm.

Bundles formed by a few microtubules. Even in oriented thin sections obtained from standard macroplasmodia, the overall structure of the cytoplasmic microtubule network strongly depended on the plasmodial area observed. But, perhaps due to the complexity of the microtubule network and the morphological plasticity of the plasmodium, no clear location of the structures playing a role in the nucleation of cytoplasmic microtubules was found.

Overall structure of the cytoplasmic microtubule network

In most areas, the elongated structures immunolabelled by the anti-tubulin antibodies constituted a very indis-
constituted of 2–8 microtubules; (•) theoretical percentage of being either isolated or part of a microtubule bundle. Fig. 4. Distribution of microtubules in cytoplasmic Poisson distribution: \( y = m^r \cdot e^{-m} \cdot r! \), \( y \) being the probability of observing a bundle with \( r \) microtubules, \( m \) the mean of the distribution. Microtubule bundles and isolated microtubules were recorded by electron-microscopic observation of thin sections obtained from interphase standard macroplasmodia; 311 bundles corresponding to a total of 647 microtubules were recorded. Two microtubules were considered to be associated when the distance between them was less than or equal to the external diameter of a microtubule.

Distinct meshwork (Fig. 1A and D, covering areas about 50 and 20 \( \mu \)m long). There was no obvious relationship between the density of cytoplasmic microtubules and the presence or absence of nuclei; microtubules were abundant in plasmodial areas containing nuclei (Fig. 1A and B) as well as in areas devoid of nuclei (Fig. 5A and B). Although some cytoplasmic microtubules turned around a nucleus (Fig. 5C and D), we have never observed microtubules interacting directly with nuclei.

Plasmodia exhibit different morphological areas such as large homogeneous parts, growing edges at the periphery of the plasmodial mass and cytoplasmic veins of various sizes (Sauer, 1982). In large homogeneous areas (Fig. 1D and e) and growing edges (Fig. 5G) cytoplasmic microtubules did not exhibit any particular orientation. They showed an apparent length varying from 2 to 10 \( \mu \)m. In contrast, in most of the cytoplasmic veins, the microtubules were generally oriented parallel to the long axis of the veins (Fig. 5E) and they reach lengths of as much as 20 \( \mu \)m. In all cases some microtubules seemed to follow the membrane between the two interconnecting veins (Fig. 5F), in agreement with electron-microscopic observations. In some large differentiated veins microtubules present in the central core of the vein were more or less parallel to the vein axis (Fig. 5H) and their length reached 60 \( \mu \)m. In contrast, microtubules present in the peripheral zone of the vein were shorter (25 \( \mu \)m) and presented no preferential orientation, although they were more or less oriented perpendicular to the edge of the vein.

Observations performed by confocal laser microscopy demonstrated that cytoplasmic microtubules, although more abundant near the upper surface, were present in all the thickness of the plasmodium (Fig. 6). Moreover, they were not necessarily parallel to the substratum, and extended over several \( \mu \)m in depth. Thus, as shown by both confocal microscopy and electron microscopy, the plasmodial cytoplasmic microtubules constitute a three-dimensional network.

Discussion

Identification of cytoplasmic microtubules in Physarum plasmodia is supported, first by ultrastructural characteristics such as length, inner and outer diameters, number of protofilaments, immunogold labelling with antibody specific for tubulin chains terminated by an aromatic amino acid; second by immunofluorescence with antibodies showing different specificities towards \( \alpha \) and \( \beta \)-tubulin isotypes; and third by the reversible susceptibility of this network to cold treatment and to two chemically distinct microtubule poisons.

The presence of microtubules in the cytoplasm of Physarum plasmodia contradicts previous observations performed both by electron microscopy and immunofluorescence (Havercroft and Gull, 1983; Solnica-Krezel et al.)
1990; Porter et al. 1965). It is likely that the absence of plasmodial cytoplasmic microtubules, repeatedly reported, is not due to their absence in the strains that have been previously used, since we observed a microtubular network in several distinct Physarum plasmodial strains. The difficulties of preserving cytoplasmic micro-

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tubules in large standard plasmodia (Dugas and Bath, 1962; Rhea, 1966) could explain the failure of previous electron-microscopic studies. The short permeabilization of plasmodia prior to their fixation with glutaraldehyde seems a requisite for good preservation of microtubules both in the cytoplasm and in mitotic nuclei. Using this procedure, images of mitotic nuclei showed a larger number of microtubules than those obtained after fixation without previous permeabilization (Moisand and Wright, unpublished observations). The reported absence of cytoplasmic microtubules by immunofluorescent staining with anti-tubulin antibodies is puzzling, since cytoplasmic microtubules have been carefully searched by different procedures (Havercroft and Gull, 1983). The disruption of the microtubule network by mechanical shearing during preparation of smears did not prevent the observation of cytoplasmic microtubules in standard plasmodia (Fig. 1H). The fixation with glutaraldehyde after gentle lysis in a microtubule-stabilizing medium permitted the observation and the immunolabelling of cytoplasmic microtubules by electron microscopy in both thin (Fig. 3B and D) and standard plasmodia (Fig. 3A, C and E). However, fixation with aldehydes (1.5 % glutaraldehyde or 3.7 % formaldehyde, for example) has not been successful in enabling us to observe the cytoplasmic microtubule network by immunofluorescence, although electron microscopy showed that the same antibodies reacted with well-preserved microtubules after fixation with glutaraldehyde. We observed no cytoplasmic microtubules when the fixation with aldehydes was either preceded by a gentle lysis in various microtubule-stabilizing media or followed by a post-fixation with methanol. Cytoplasmic microtubules were repeatedly observed in semi-thin sections and in smears obtained from standard plasmodia, but a high level of background fluorescence was always present and prevented a clear observation of the microtubule network (Fig. 1G and H). Although more limited in thin plasmodia (Fig. 1D and E), the same difficulty was noticeable, especially when we followed the modified protocol necessary for using the TPH 4 monoclonal antibody (Fig. 1F). This background of fluorescence was probably not due entirely to the diffuse fluorescence of out-of-focus immunolabelled microtubules. Diffuse fluorescence could be observed also in thin plasmodia of 4–5 μm in thickness with a scanning laser microscope as shown by the fluorescent dots that remained present in the three-dimensional reconstructions despite the efficient threshold image treatment that was applied to each image before three-dimensional reconstruction (Fig. 6). Thus, we suggest that the preparative procedure that we have used for successful immunofluorescence labelling of cytoplasmic microtubules could reduce the intrinsic background fluorescence of plasmodia. However, it must be considered that additional factors could interfere with the observation of cytoplasmic microtubules. Although cytoplasmic microtubules have been repeatedly immunolabelled with three distinct anti-tubulin monoclonal antibodies, the efficiency of each antibody to label cytoplasmic microtubules clearly depended on the fixation procedure applied to thin plasmodia and to standard plasmodia. Although immunolabelling with the YL 1/2 and KMX 1 monoclonal antibodies could be observed after a fixation in cold methanol (Fig. 1D and E), this procedure was not adequate for the observation of cytoplasmic microtubules with the TPH 4 monoclonal antibody (not shown). With this anti-tubulin antibody, the best results were obtained when the fixation by cold methanol was preceded by a gentle permeabilization in a microtubule-stabilizing medium (Fig. 1F).

Cytoplasmic microtubules have been reported in the multinucleate protoplasmia of the myxomycete *Echinostelium minutum* (Hinchee, 1976), which remains microscopic throughout its entire existence (Gray and Alexopoulos, 1968), reaching a maximum size of 250 μm (Hinchee, 1976) and representing the most primitive type of plasmodium (Alexopoulos, 1960; Alexopoulos, 1962). In its early stages the plasmodium of *Physarum* is hardly distinguishable from a protoplasmodium at the same stage of development (Gray and Alexopoulos, 1968). Similarly, in developing small *Physarum* plasmodia it has been recently reported from immunofluorescence studies that 'microtubules formed a very fine and uniform meshwork' that 'could be detected even in some plasmodia containing more than 100 nuclei' (Solnica-Krezel *et al.* 1990). Solnica-Krezel *et al.* concluded that 'cytoplasmic microtubules disappear, so that in the mature plasmodium the only microtubular structure detected is the closed mitotic spindle'. However, it cannot be argued that the thin
Physarum plasmodia that have been used in part of this study are not relevant to the ultrastructure of the usual synchronous giant plasmodia. First, in contrast with protoplasmidia, thin plasmodia possessed the overall structure of phaneroplasmidia as shown by the presence of differentiated protoplasmic veins (Fig. 5H). Second, thin plasmodia were much larger than protoplasmidia and contained on average $8.8 \times 10^9$ to $1.8 \times 10^{10}$ nuclei. Similarly, it could also be argued that the procedure used to flatten plasmodia between two pieces of agar could induce some stress or lead to early plasmodial ageing. Thus it can be proposed that in these conditions the assembly of cytoplasmic microtubules corresponds to a pre-differentiated state leading to sperulation or sporulation. This is probably not the case, since the procedure used to flatten plasmodia mimics the conditions that are encountered by small plasmodia in decaying forest substrata. Moreover, no morphological signs support the hypothesis that flattened plasmodia were entering in a pre-differentiated state. First, sandwiched plasmodia did not show the compartmentalization that precedes the formation of sperules. Second, all experiments were performed in the dark and did not permit the induction of sporulation. Third, although a shortage of oxygen could act in preventing mitosis, the third mitosis occurred normally and synchronously in thin plasmodia. Moreover, cytoplasmic microtubules were not restricted to thin plasmodia. They were observed by immunofluorescence in standard plasmodia during the third cell cycle by two different procedures: in thin sections (Fig. 1G) and in smears (Fig. 1H). Although in both cases it was not possible to observe the overall disposition of the cytoplasmic microtubule network, cytoplasmic microtubules were visible, in agreement with electron-microscopic observations made in both standard (Fig. 3A, C and E) and flattened plasmodial pieces (Fig. 3B and D). These observations demonstrate that cytoplasmic microtubules are present both in standard plasmodia and in flattened plasmodial pieces.

Thus, thin plasmodia constitute a convenient model for the observation of both the microtubule (this report) and the microfilament network (Kamiya and Kuroda, 1965; Naib-Majani et al. 1983; Naib-Majani et al. 1982).

In contrast with the oversimplified view that has been widely accepted, our demonstration of the presence of a cytoplasmic microtubule network in Physarum plasmodia raises several problems of general interest. First, the ultrastructural location of the cytoplasmic microtubules and microfilaments suggests that they form two distinct cytoskeletal elements. Thus it is necessary to determine the relationships between them and to discover their respective roles in protoplasmic streaming and cell organelle movement (Porter et al. 1965). Second, our observations suggest the need to reinvestigate several questions that have been addressed to Physarum plasmodia, such as the use of the various tubulin isofoms (Burland et al. 1983; Paul et al. 1987; Burland et al. 1988), the transient synthesis of tubulin in late $G_2$-phase (Laffter et al. 1981; Carrino and Laffter, 1985), the arrest of tubulin degradation $3 \text{h}$ after mitosis (Carrino and Laffter, 1985; Duccommun and Wright, 1989), and the transient phosphorylation of a microtubule-binding protein in late $S$-phase (Albertini et al. 1990). Third, the observation of cytoplasmic microtubules in Physarum plasmodia raises questions concerning the presence of cytoplasmic microtubule organizing centers, as well as the dynamics and turnover of microtubules in a constantly moving cytoplasm, both in interphase and in mitosis when the protoplasmic streaming seems to be modified (Guttes and Guttes, 1963; Sachsenmaier et al. 1973; Kessler and Lathwell, 1979; Nations et al. 1981; Kessler et al. 1981).

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References


Physarum plasmodia contain cytoplasmic microtubules