Cell Type-dependent Expression of Tubulins in *Physarum*

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ABSTRACT Three α-tubulins and two β-tubulins have been resolved by two-dimensional gel electrophoresis of whole cell lysates of *Physarum* myxamoebae or plasmodia. Criteria used to identify the tubulins included migration on two-dimensional gels with myxamoebal tubulins purified by self-assembly into microtubules in vitro, peptide mapping with *Staphylococcus* V8 protease and with chymotrypsin, immunoprecipitation with a monoclonal antibody specific for β-tubulin, and, finally, hybrid selection of specific mRNA by cloned tubulin DNA sequences, followed by translation in vitro. Differential expression of the *Physarum* tubulins was observed. The α1-and β1-tubulins were detected in both myxamoebae and plasmodia; α2 and β2 were detected only in plasmodia, α3 was detected only in the myxamoebal phase, and may be specific to the flagellate. Observation of more tubulin species in plasmodia than in myxamoebae was remarkable; the only microtubules detected in plasmodia are those of the mitotoic spindle, whereas myxamoebae display cytoplasmic, centriolar, flagellar, and mitotic-spindle microtubules. In vitro translation of myxamoebal and plasmodial RNAs indicated that there are distinct mRNAs, and therefore probably separate genes, for the α1-, α2-, β1-, and β2-tubulins. Thus, the different patterns of tubulin expression in myxamoebae and plasmodia reflect differential expression of tubulin genes.

Microtubules are filamentous structures that are implicated in several functions in eucaryotic cells, including nuclear division, motility, and the determination of cell shape. They are assembled from heterodimeric subunits of α- and β-tubulin polypeptides. The α- and β-tubulins of vertebrates have ~40–50% homology in amino acid sequence (19, 31, 39), and exhibit similar but distinct electrophoretic mobilities (e.g., 22).

In several eucaryotes, electrophoretic heterogeneity of both α- and β-tubulins has been observed. Within a single organism, tubulin heterogeneity can be detected at several levels. At one level, different tissues or cell types may express different tubulins. In *Drosophila*, for example, one β-tubulin subunit is found in most somatic tissues, whereas an electrophoretically distinct β-tubulin is found only in the testis (18). At another level, different organelles may be assembled from different tubulins. In the sea urchin, differences have been detected between the tubulins of the flagella, the cilia, and the cytoplasm or mitotic spindle (1, 36). In the unicellular algae *Polytomella* and *Chlamydomonas*, differences have been observed between the tubulins of the cytoplasmic microtubules and the flagellar microtubules, two organelles within the same cell type (4, 21, 24, 25). Perhaps the most extensive heterogeneity is observed in tubulins purified from mammalian brain, where 17 different polypeptides have been resolved by isoelectric focusing (14). However, in this case, it is not yet known how the different isoforms are distributed over the different cell types and organelles, although at least one class of sympathetic neurone expresses most of the tubulin isoforms in cell culture (15).

These observations are consistent with the multitubulin hypothesis, which proposed that different microtubular structures are assembled from different tubulins, and thus that the tubulin heterogeneity has functional significance (11). However, analysis of the effects of mutations in the gene coding for the testis-specific β-tubulin in *Drosophila* indicates that this subunit has multiple functions during spermatogenesis, including meiosis, nuclear shaping, and axoneme assembly (17). It thus remains uncertain whether tubulin heterogeneity is a reflection of strict functional specialization.

The myxomycete *Physarum polycephalum* displays several different microtubular structures in the different phases of its life cycle. The uninucleate myxamoebae exhibit cytoplasmic, mitotic-spindle, and centriolar microtubules; under moist, non-nutrient conditions, myxamoebae transform into flagellates, each cell developing two microtubule-based flagella (33). Myxamoebae can also develop, usually sexually, into multinucleate, syncytial plasmodia. Microtubules are present in the

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plasmodia only during the synchronous, intranuclear mitosis; cytoplasmic, centriolar, and flagellar microtubules have never been observed (33). The appearance of microtubules during plasmodial mitosis follows the pronounced rise in tubulin synthesis late in the G2 phase of the cell cycle (20, 37). In contrast, microtubules are present throughout the cell cycle of the myxamoebae. The myxamoeba and the plasmodium of *Physarum* are thus two cell types that exhibit substantial differences in their microtubular structures. Recent progress in the purification and characterization of self-assembled tubulin from *Physarum* myxamoebae greatly facilitates investigations of *Physarum* tubulins (6, 7, 33, 34).

This study reports the identification of tubulins in whole cell lysates of *Physarum* myxamoebae and plasmodia, and shows that differential expression of tubulins occurs in these two phases of the life cycle.

MATERIALS AND METHODS

Strains and Culture Conditions: Plasmodia of strain M_3CV (27) were maintained as microplasmodia in simplified soy medium (SSM)[1] (20). Synchronous plasmodia were prepared by inoculation of microplasmodia (concentrated by centrifugation) onto double layers of Schleicher & Schuell, Inc. (Keene, NH) No. 576 filters, which were underlayered with SSM (15 ml/9-cm petri dish) as soon as excess medium had soaked into the filter; no starvation period was used.

Myxamoebae of strain CLd (10) were cultured on formalin-killed *Escherichia coli* on 0.1% liver infusion agar (Oxoid). Strain CLd-AXE myxamoebae, which are derived from strain CLd (23), were cultured in SSM in shake flasks. All incubations were at 26°C.

In Vivo Labeling of Polypeptides: Different labeling protocols were used for myxamoebae and plasmodia because of their different growth requirements.

To label plasmodial polypeptides, we cut a 7-mm disk of plasmodium growing on filter paper from a large (~70 mm diam) plasmodium and transferred to filters moistened with SSM. Approximately 50 μCi (5 μl) of [^{35}S]methionine (SJ204; Amersham Corp. Arlington Heights, IL) was placed on top of the plasmodial disk at ~1 h before the second mitosis (MII) following inoculation, since microtubule proteins are synthesized maximally around this time. When MII occurred in the larger parent plasmodium, the smaller disk of labeled plasmodium was harvested with a spatula and prepared immediately for two-dimensional (2D) gel electrophoresis.

Most *Physarum* myxamoebal strains, including the CLd strain used in this work, are unable to grow axenically and are routinely cultured on bacterial lawns. Myxamoebae were labeled by culturing for several days on formalin-killed *E. coli* that had previously been labeled with [^{35}S]sulphate (NEX-041; New England Nuclear, Boston, MA) in morpholine propane sulfonic acid medium (29). Labeled myxamoebae were harvested at room temperature in water by scraping them off the plates with a glass spreader; this step allows a proportion of the myxamoebae to transform into flagellates. Myxamoebae were centrifuged free of bacteria, then prepared for 2D gel electrophoresis. When unlabeled myxamoebae were mixed with labeled *E. coli* just before centrifugation, no labeled proteins were observed on the resulting 2D gels, which indicates that the washing procedure was removing the *E. coli* effectively. Turnock et al. (38) have previously established that *E. coli* proteins do not contribute significantly to the myxamoebal 2D gel patterns when the myxamoebae are washed free of bacteria, which indicates that ingested, undigested *E. coli* are not present at a significant level.

Since myxamoebae and plasmodia were usually labeled under different conditions, it was important to carry out a control experiment in which the myxamoebae and plasmodia were labeled under identical conditions. For this control, myxamoebae and plasmodia were labeled with killed ^{35}S-labeled *E. coli* on the surface of agar plates made with 1/10-strength SSM. Incubation was continued for 4 d; then the cultures were harvested in water and centrifuged free of bacteria. The myxamoebal and plasmodial 2D gel patterns observed under the more efficient labeling protocols were preserved in this control experiment. Thus, the differences observed between 2D gel patterns of myxamoebal and plasmodial polypeptides are not artifacts of the different labeling protocols.

[1] *Abbreviations used in this paper:* 1D, one-dimensional; 2D two-dimensional; DBM, diazobenzyloxymethyl; SSM, simplified soy medium.

Sample Preparation for Electrophoresis: For 2D gels, one 7-mm-diam plasmodium or 2–5 × 10^7 myxamobae were lysed by three cycles of freeze-thaw in 50 μl lysis buffer (1% Nonidet P40, 20 mM Tris, pH 7.6, 5 mM $MgCl_2$, and 50 μg/ml leupeptin [Sigma Chemical Co., St. Louis, MO]). Next, 5 μl nuclease solution (2 mg/ml DNaseI, 1 mg/ml RNase A, [both from Worthington Biochemical Corp., Freehold, NJ], and 50 μg/ml leupeptin) was added. After 15 min on ice, 50 mg urea (Bethesda Research Laboratories, Gaithersburg, MD) and 100 μl sample buffer (9.5 M urea, 5% 2-mercaptoethanol, 2% Nonidet P40, and 2% LKB ampholines) were added. After one further cycle of freeze-thaw, samples were either loaded onto gels or stored at −70°C. In vitro translation products were first precipitated with 5 vol of cold acetone and resuspended in lysis buffer, from which point samples were processed as for in vivo-labeled material.

For one-dimensional (1D) gels to resolve purified myxamoebal proteins, lyophilized proteins were dissolved in water then diluted fourfold in SDS sample buffer and boiled before loading.

Electrophoresis: 2D gel electrophoresis was carried out by the original procedure (30) with the following modifications: (*a*) the mixture of ampholines used was a 3:2 ratio of pH 3.5–10/pH 5–7; (*b*) the prefocusing run was omitted, and instead focusing was begun, with samples loaded, at a limited 0.17 mA per tube gel; when the voltage increased sufficiently, electrophoresis was continued at a constant 400 V for a total of 16.5 h from the start of electrophoresis; focusing was then continued for an additional 3 h at 800 V; (*c*) a 10% polyacrylamide gel was used for the second dimension.

To determine whether particular electrophoretic species among different samples migrated to the same 2D gel position, fluorograms or stained gels from the same electrophoresis run were compared by superposing the major actin spot and other spots in the actin-tubulin region of the gels.

1D electrophoresis to resolve purified proteins was essentially the same as the second dimension of the 2D gels.

Peptide Mapping: Peptide mapping of proteins in gel slices was carried out as described by Cleveland et al. (8). Individual spots on 2D gels were excised for proteolysis only when the fluorograms indicated that they were clearly resolved from adjacent spots. Gel slices were placed directly in sample wells of peptide mapping gels, without elution of protein. *Staphylococcus aureus* V8 protease (Miles Laboratories Inc., Elkhart, IN) was used at a concentration of 0.2 μg/lane. Chymotrypsin (Sigma Chemical Co.) was used at a concentration of 0.05 μg/lane. For each enzyme, proteolysis was performed by stopping electrophoresis for 10 min when samples had been run into the stacking gel; peptides were then resolved by electrophoresis through the 15% acrylamide separating gel and detected either by silver staining (41) or by fluorography of gels treated with EN^{3}HANCE (New England Nuclear). When silver staining and fluorography were performed on the same gel, the silver was first destained with Kodak fixer before fluorography.

Immunoprecipitation: Plasmodial polypeptides were first labeled with [^{35}S]methionine late in the G2 phase of the cell cycle as described above. Immunoprecipitation of the labeled polypeptides was carried out as described by Mose-Larsen et al. (28) using anti-β-tubulin monoclonal antibody DM1B (2). DM1B hybridoma ascites fluid was a generous gift of S. H. Blose (Cold Spring Harbor Laboratory).

Isolation of RNA: All glassware was baked at 210°C for 12 h; all solutions were filtered (Nalgene, 0.45 μm pore diam), and some solutions were treated with 0.1% diethylpyrocarbonate before autoclaving. RNA was isolated by a modification of the method of Kaplan et al. (16). Briefly, to 1 × 10^9 axenic amoebae or 0.5–1.0 g of synchronous macroplasmodium, 9 ml of lysis buffer (5.0 M guanidine thiocyanate [Fluka Chemical Corp., Hauppage, NY], 50 mM Tris HCl [pH 7.6], 10 mM EDTA, and 5% 2-mercaptoethanol) was added and immediately homogenized with a Polytron (Brinkmann Instruments, Inc., Westbury, NY) for 30 s. The homogenate was made 4% (wt/vol) with respect to *N*-lauroylsarcosine, and solid CsCl (Schwarz/Mann, Spring Valley, NY) was added to 0.15 g/ml. After gentle mixing, the suspension was centrifuged at 8,000 *g* for 10 min and the supernatant passed through Miracloth (Chicopee Mills, Inc., Milltown, NJ). The solution was layered over a 2.5-ml cushion of 5.7 M CsCl (density 1.705 g/cm^3) and 0.1 M EDTA (pH 7.6), and centrifuged in an SW41 rotor (Beckman Instruments, Inc., Palo Alto, CA) at 36,000 rpm for 24 h at 20°C. After centrifugation, the supernatant was removed by aspiration to just below the polysaccharide band (~1 cm from the bottom); the tube was inverted to drain the remaining liquid and the bottom 1 cm of the tube was cut off with a razor blade. The RNA pellet was dissolved in water, made 0.1 M LiCl, and precipitated with 2 vol of ethanol. Poly A-containing RNA was isolated by chromatography with poly (U)-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ).

Hybrid Selection and In Vitro Translation of RNA: Hybrid selection was performed as described by Miller et al. (26), using the diazobenzyloxymethyl (DBM) paper protocol. The DNA immobilized to DBM paper were from (*a*) an α-tubulin cDNA clone isolated from *Physarum* plasmodial

RNA (T. Schedl, unpublished); (*b*) a chick β-tubulin cDNA clone, pT2, a generous gift of D. Cleveland (9), and, on a separate filter, a *Drosophila* β-tubulin genomic clone, pDTB4, a generous gift of S. Natzle (35); (*c*) a 1.8-kb fragment containing the entire coding sequence from the actin genomic clone λDmA2, a generous gift of E. Fyrberg (12, 13); (*d*) pBR322 vector. To each filter was hybridized 2 μg of poly A-containing RNA isolated from late G2 phase plasmodia. For the α-tubulin and actin hybridizations, the filters were washed 10 times at high stringency (30 mM Na^+ at 37°C) after hybridization. Under these conditions, when a control pBR322 filter was used, only translation products endogenous to the wheat germ system were detected (data not shown). DBM filters containing chick β-tubulin sequences were combined with filters containing *Drosophila* β-tubulin sequences throughout the hybrid selection. After hybridization, these filters were washed under low stringency (300 mM Na^+ at 37°C). When the low-stringency wash was used for pBR322 selection, polypeptides in addition to the endogenous products were detected. However, β-tubulins were not among them (data not shown). RNA eluted from each hybrid selection, or total RNA from plasmodia or myxamoebae, was translated in the wheat germ cell-free system (32) as described by Boston et al. (3).

RESULTS

Characterization of Self-assembled Tubulin from Myxamoebae

A useful functional definition of tubulins is the ability to assemble into microtubules in vitro (40). Myxamoebal tubulin purified by rounds of self-assembly and disassembly in vitro has already been well characterized (6, 7, 33, 34). 1D PAGE resolves this preparation into two bands, α- and β-tubulin. Comparison of these two *Physarum* subunits with mammalian brain tubulins by peptide mapping has shown that the *Physarum* α-tubulin migrates faster than the *Physarum* β-tubulin, the reverse of the situation in mammalian brain (6, 33). The identity of *Physarum* α-tubulin in the self-assembled material has also been confirmed immunologically (5).

As a standard for this work, CLd-AXE myxamoebal tubulin purified by two rounds of self-assembly in vitro was used (33). In addition to α- and β-tubulin, this preparation contains actin, which serves as a useful marker for 2D gel electrophoresis; the resolution of these three polypeptides on 2D gels is shown in Fig. 1 *a*. The purified myxamoebal α- and β-tubulin electrophoretic species will be termed α1- and β1-tubulin, respectively (Fig. 1 *a*). Throughout this paper, resolvable electrophoretic species are given separate designations (α1, α2, etc.); it should be noted, however, that a single electrophoretic species may consist of more than one type of tubulin polypeptide.

Comparison of the Electrophoretic Patterns of Proteins Labeled During Growth of Myxamoebae and Plasmodia

The 2D electropherogram of myxamoebal proteins labeled with [^{35}S]-*E. coli* (see Materials and Methods) is shown in Fig. 1 *b*. Labeled myxamoebal polypeptides that migrate to the same positions as the purified α1- and β1-tubulins are detectable, and, in addition, another species, α3-tubulin, is observed (Fig. 1 *b*). The identification of the α3-tubulin is presented below. The absence of α3 among the purified myxamoebal tubulins is probably related to the growth conditions preceding purification (see below, In Vitro Translation of Myxamoebal RNA).

The 2D gel electropherogram of plasmodial proteins labeled with [^{35}S]methionine in the late G2 phase of the cell cycle is shown in Fig. 1 *c*. Labeled species that migrate to the same positions as the myxamoebal α1- and β1-tubulins are again present. In addition, two other tubulins, α2 and β2, were detected (Fig. 1 *c*); the identification of these polypeptides is presented below. All four plasmodial tubulins are synthesized specifically in the last 2–3 h of the synchronous cell cycle, before mitosis (unpublished observations). Other polypeptides in the tubulin region of the gels are synthesized throughout the cell cycle (not shown). The differences observed between the myxamoebal and plasmodial tubulins are not artifacts of the different labeling protocols, since these differences are preserved when plasmodia are labeled with [^{35}S]-*E. coli* (see Materials and Methods).

Although the data presented compare the polypeptides of a haploid myxamoebal strain (CLd) with those of a diploid plasmodial strain (M_3CV) that is derived from a natural isolate (27), the α2- and β2-tubulins are not simply electrophoretically detectable polymorphisms, since the same plasmodial tubulins were detected in the CLd haploid plasmodia (not shown).

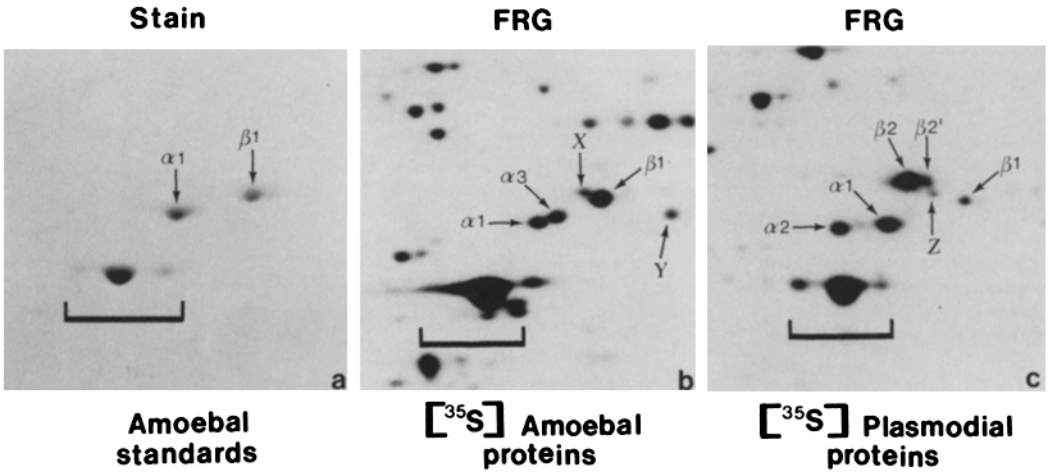

FIGURE 1 Proteins separated by 2D gel electrophoresis. (a) Purified myxamoebal microtubule proteins, detected by staining with Coomassie Blue. (*b*) Myxamoebal proteins, labeled in vivo with ^{35}S-labeled *E. coli*, detected by fluorography. (c) Plasmodial proteins, labeled in vivo with [^{35}S]methionine, detected by fluorography. Only the actin-tubulin region of each gel is shown. Isoelectric focusing is from left (basic) to right (acidic), SDS polyacrylamide separation is from top to bottom. The horizontal bar delineates the actin region. *α1*, *α2*, and *α3*, α-tubulins. *β1* and *β2*, β-tubulins.

Identification of Tubulins by Peptide Mapping

1D peptide mapping (7) was used to determine which of the radiolabeled polypeptides resolved on 2D gels were likely to be tubulins. To establish identities between the purified tubulins and the labeled polypeptides, the purified myxamoebal proteins were first resolved in 1D gels and excised separately. Then, one labeled protein, excised from a 2D gel, and one of the two purified tubulins were placed together in a sample well of a 15% polyacrylamide gel, and peptide mapping with *Staphylococcus* V8 protease was performed (8). The peptides from the purified tubulin were detected by silver staining, and those of the labeled protein were detected by fluorography (Fig. 2). This procedure ensured that the proteolysis conditions were the same for both the unlabeled purified tubulin and the much less abundant labeled polypeptide.

One of the labeled plasmodial species migrates on 2D gels to the position of the purified myxamoebal α1-tubulin (Fig. 1); the V8 peptide maps (Fig. 2; see also reference 5) suggest that this labeled plasmodial species is also α-tubulin. The most strongly labeling plasmodial species in the β-tubulin region of the 2D gels (β2 in Fig. 1) was compared with the purified myxamoebal β1-tubulin by V8 proteolysis (Fig. 2); although these two species did not display identical peptide maps, the similarities between them are striking, considering that one of the peptide maps was detected by silver staining, whereas the other was detected by fluorography of methionine-labeled protein. The strongly labeling plasmodial species was designated β2-tubulin (Fig. 1).

The labeled plasmodial α1- and β2-tubulins were then used as standards for peptide mapping of other labeled polypeptides. The V8 protease peptide maps shown in Fig. 3 *a* indicate that there are two α-tubulin and two β-tubulin electrophoretic species detectable in plasmodia, labeled α1-, α2-, β1-, and β2-tubulin in Fig. 1 *b*. As expected, the weakly labeling plasmodial polypeptide that migrates on 2D gels to the position of the standard myxamoebal β1-tubulin also resembles β-tubulin (Fig. 1 *b*; see also reference 5). The V8 protease-generated peptide maps of the in vivo-labeled myxamoebal polypeptide species that co-migrate on 2D gels with the purified α1- and β1-tubulins (Fig. 3 *b*) are similar to the peptide maps of the labeled plasmodial α1- and β1-tubulins, respectively (Fig. 3 *a*), which suggests their identity as tubulins. An additional tubulin species, α3, was detected among labeled myxamoebal polypeptides (Figs. 1 *b* and 3 *b*), but was not detected in plasmodia. The α2- and β2-tubulins of the plasmodia were not detected in myxamoebae (Fig. 1). The V8 peptide maps of two other myxamoebal polypeptides in the tubulin region of the 2D gels (spots *x* and *y* in Fig. 1 *c*) showed some differences from the α- and β-tubulins (Fig. 3 *b*).

Peptide mapping with a second protease, chymotrypsin, confirmed the similarity between α1- and α3-tubulins of the myxamoebae, and between the α1- and α2-tubulins of the plasmodia (Fig. 4 *a*). The myxamoebal β1-tubulin and the plasmodial β1- and β2-tubulins gave chymotrypsin-generated peptide maps similar to one another and distinct from the α-tubulins (Fig. 4 *b*). The chymotrypsin peptide map of plasmodial polypeptide Z (Fig. 1 *c*) differed from the peptide maps of the α- and β-tubulins (Fig. 4 *b*). This result was expected, since the spot *z* polypeptide is labeled continuously throughout the plasmodial cell cycle, whereas the tubulins are labeled specifically late in the G2 phase.

The plasmodial β2-tubulin appears to consist of at least one major and one minor component (β2 and β2′, respectively, in Fig. 1 *c*) that are incompletely resolved. The plasmodial α1-tubulin also appears complex in some electrophoretic runs, especially in 2D gels of in vitro translation products (see below, In Vitro Translation of Total Cellular RNA). Peptide mapping of the α1′ and β1′ spot regions, however, failed to

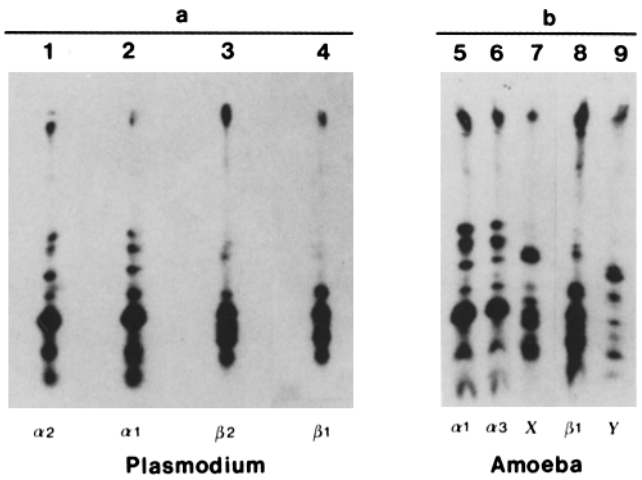

FIGURE 3 Peptide mapping of radiolabeled proteins using *Staphylococcus* V8 protease. (*a*) Lanes *1–4*: plasmodial tubulins. (*b*) Lanes *5*, *6*, and *8*: myxamoebal tubulins. Lanes *7* and 9: myxamoebal proteins x and y (see Fig. 1). Labeled polypeptides were first resolved on 2D gels and detected by fluorography, and then individual spots were excised from dried gels. Excised gel pieces were then placed directly in sample wells of slab gels, and proteolysis was performed after running the samples into the stacking gel (8). Note that peptide maps of the same proteins in Figs. 2 and 3 may not be identical, since the addition of a gel slice containing purified tubulin to sample wells (Fig. 2) will substantially elevate the substrate concentration for the protease.

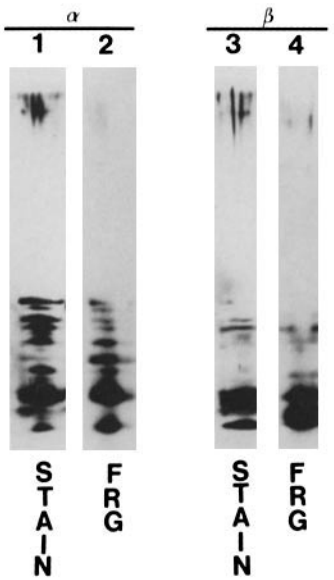

FIGURE 2 Peptide mapping of purified (self-assembled) myxamoebal tubulins and radiolabeled plasmodial proteins with *Staphylococcus* V8 protease. Lanes *1* and *2*: excised gel slices containing the standard myxamoebal α1-tubulin and the radiolabeled plasmodial protein that co-migrates with it on 2D gels were transferred to a sample well and peptide mapped in the same lane of the gel. Lane *1*: silver stain, showing a peptide map of the standard myxamoebal α1-tubulin. Lane *2*: fluorogram of same lane as *1*, showing a peptide map of the labeled plasmodial protein that co-migrates with the purified α1-tubulin on 2D gels. Lanes *3* and *4*: excised gel slices containing the standard myxamoebal β1-tubulin and a radiolabeled plasmodial protein (β2 in Fig. 1 *b*) were peptide mapped in the same lane of the gel. Lane *3*: silver stain, showing a peptide map of the standard myxamoebal β1-tubulin. Lane *4*: fluorogram of same lane as *3* showing a peptide map of plasmodial β2-tubulin.

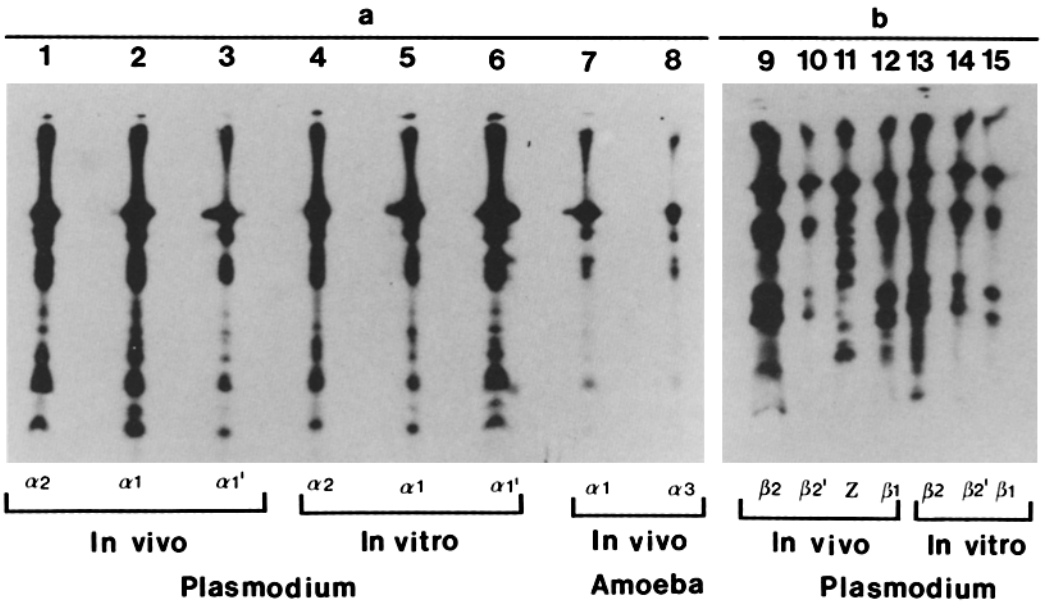

FIGURE 4 Peptide mapping of radiolabeled proteins using chymotrypsin. (*a*) α-tubulins, lanes *1–3*: in vivo-labeled plasmodial tubulins. Lanes *4–6*: in vitro translation products of plasmodial RNA. Lanes *7–8*: in vivo-labeled myxamoebal tubulins. (*b*) β-tubulins, lanes *9*, *10*, and *12*: in vivo-labeled plasmodial tubulins. Lanes *13–15*: in vitro translation products of plasmodial RNA. Note that protein Z, lane *11* (see Fig. 1), shows differences from both α- and β-tubulin. Peptide mapping was performed as described in the legend to Fig. 3.

detect any major contamination by non-tubulin proteins (Fig. 4).

Laffler et al. (20) have already shown that the plasmodial β2-tubulin (which they referred to as "spot P") co-assembles into microtubules in vitro when pig brain tubulin is used as carrier. Subsequently, Chang et al. (5) presented peptide mapping data suggesting that "spot P" was unlike myxamoebal α- or β-tubulin. This conclusion may have been reached erroneously by using different proteolysis conditions for the standard myxamoebal β-tubulin and the labeled plasmodial species. Their analysis may also have been complicated by failure to resolve the polypeptide Z (Fig. 1) that migrates on our gels between β1- and β2-tubulin: compare Fig. 1 of Chang et al. (5) with our Fig. 1.

Immunoprecipitation by an Anti-β-Tubulin Monoclonal Antibody

Rigorous confirmation of the identities of the *Physarum* β-tubulins was sought to dispel any possible doubts raised by: (*a*) the suggestion by Chang et al. (5) that the species identified here as β2-tubulin was unlike β-tubulin, and (*b*) the slight differences observed between the peptide maps of the β1- and β2-tubulin species. The monoclonal antibody DM1B, raised against chick tubulin and specific to the β-subunit (2), enabled us to identify *Physarum* β-tubulins by immunoprecipitation.

Disks of plasmodia late in the G2 phase of the cell cycle were labeled with [^{35}S]methionine. Immunoprecipitation of labeled proteins with the DM1B antibody was carried out as described by Mose-Larsen et al. (28); immunoprecipitated proteins were then resolved on 2D gels. Fig. 5 shows that both the β1- and β2-tubulins are precipitated by DM1B, to the exclusion of all other polypeptides in the tubulin region of the gel. This confirms the identities of the *Physarum* β1- and β2-species as β-tubulins and suggests that polypeptide Z (Fig. 1 *c*) is not a β-tubulin.

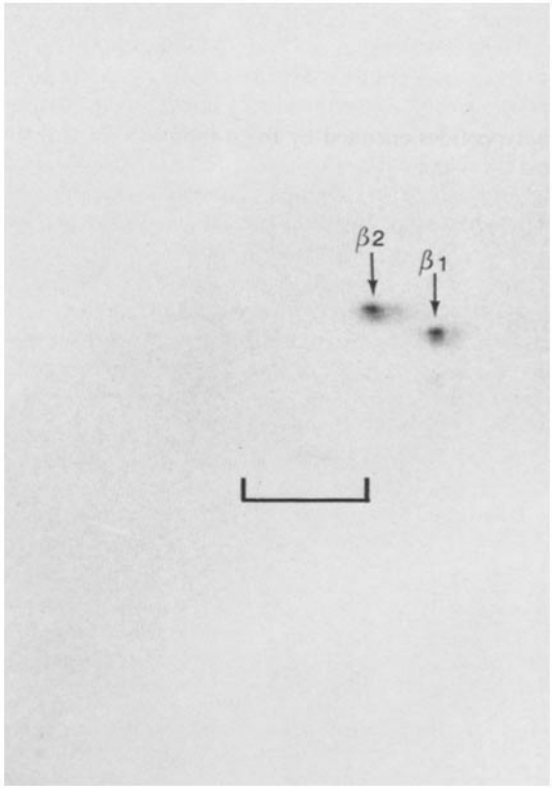

FIGURE 5 2D gel electrophoresis of proteins precipitated by the β-tubulin-specific antibody DM1B. See the legend to Fig. 1 for 2D gel conditions. Briefly, plasmodial disks labeled in the late G2 phase were boiled in SDS sample buffer (30), diluted 1:20 with 1% Triton, 1% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 50 mM Tris, pH 7.5, and cleared by centrifugation. Samples were incubated with mouse serum and protein A-Sepharose and cleared by centrifugation before incubation with DM1B ascites fluid. Protein A-Sepharose was then added for precipitation of β-tubulins.

Identification of Tubulins and Actin by Hybrid Selection

An additional method of characterizing proteins resolved by 2D gel electrophoresis is the use of identified recombinant DNA clones in hybrid selection—in vitro translation assays of mRNA.

HYBRID SELECTION OF α-TUBULIN-RELATED MRNA: A *Physarum* α-tubulin-homologous cDNA clone, Ppc-α125, (T. Schedl, unpublished data) was bound to DBM paper and hybridized to poly A^+ RNA isolated from plasmodia late in the G2 phase of the synchronous cell cycle. After washing at high stringency, the RNA selected by the hybridization was eluted and translated in vitro. Resolution of the translation products on 2D gels (Fig. 6*a*) showed that two polypeptides that migrate with α1- and α2-tubulin in parallel gels are the only species translated from the α-tubulin-related mRNA. This experiment thus confirms the identity of the α1- and α2-tubulins.

HYBRID SELECTION OF β-TUBULIN-RELATED MRNA: No homologous *Physarum* β-tubulin-cloned DNA probe is presently available, so for the hybrid selection of β-tubulin RNA, heterologous β-tubulin DNA clones from *Drosophila* and chick were bound to DBM paper and combined in the same tube. Late G2 phase plasmodial poly A^+ RNA was hybridized to these clones; the hybridization and washing were performed at low stringency (see Materials and Methods), since it is known that these heterospecific clones hybridize poorly in Southern blot experiments to *Physarum* genomic DNA (T. Schedl, unpublished data). The hybrid-selected RNA was eluted and translated in vitro, and the translation products were then resolved on 2D gels. The two most prominent polypeptides encoded by the β-tubulin-related mRNA migrated on parallel gels with β1- and β2-tubulins (Fig. 6*b*), confirming the identity of these two species. In addition, a smaller amount of α1-tubulin was also among these translation products. All other polypeptides observed among the translation products were also present among the translation products of RNA selected by the pBR322 vector DNA alone (data not shown). This experiment thus shows a preferential enrichment for β-tubulin-related mRNA; studies reported below show that when unfractionated plasmodial RNA is translated in vitro, there is much more α1-tubulin than β1-tubulin among the translation products. Short stretches of homologous sequence may have allowed sufficient hybridization to account for the translation of a trace of α1-tubulin in vitro.

HYBRID SELECTION OF ACTIN-RELATED MRNA: The actin polypeptides labeled in Fig. 1 were originally only tentatively designated as actin on the basis of 2D gel mobility (~43,000 mol wt, pI ~5.5), which is characteristic of actins from many eucaryotes, including *Physarum* (42). It is important to identify this polypeptide, as it can be used as a reference in many investigations (e.g., reference 20). To this end, late G2 phase plasmodial poly A^+ RNA was hybridized to a cloned actin DNA sequence from *Drosophila*. Two abundant actin polypeptides were observed among the in vitro translation products of this hybrid-selected RNA (Fig. 6*c*). One of these actins migrates on 2D gels to the same position as the abundant actin species observed among the in vivo-labeled polypeptides (Fig. 1), confirming the identity of *Physarum* actin on our 2D gels. The other, more basic actin translation product corresponds to a minor species among the in vivo-labeled *Physarum* polypeptides (Fig. 1).

In Vitro Translation of Total Cellular RNA

It is important to determine whether the multiple α- and β-tubulins detected arise by modification of a single gene product each for α- and β-tubulin, or whether there are multiple genes for each type of subunit. Because of the specific patterns of expression of the tubulins in myxamoebae and plasmodia, in vitro translation of RNA isolated from each of these two phases of the life cycle can be used to address this question.

IN VITRO TRANSLATION OF MYXAMOEBAL RNA: Total RNA was isolated from CLd-AXE myxamoebae grown in liquid semidefined medium; the RNA was then translated in vitro and the products were resolved on 2D gels. The only tubulin species observed among in vitro translation products were α1 and β1 (Fig. 7*a*). The identity of these tubulins was confirmed by peptide mapping (not shown). The α3-tubulin observed among polypeptides labeled in vivo was not present among the translation products of the myxamoebal RNA, which would seem to suggest that α3 is a product of posttranslational modification of α1. However, the absence of α3 was probably related to the growth conditions used preceding RNA isolation. When CLd-AXE myxamoebal proteins were labeled with [^{35}S]methionine during axenic growth, α3-tubulin was not detected. By contrast, when CLd-AXE myxamoebal proteins were labeled by growth on ^{35}S-labeled *E. coli* and then suspended in water, α3 was detected. A proportion of the myxamoebae transform into flagellates in water, whereas no myxamoebae transform in axenic medium. Expression of α3-tubulin thus correlates with the presence of flagellates. Whether the *Physarum* α3-tubulin is encoded by mRNA distinct from the α1-tubulin mRNA remains to be determined.

IN VITRO TRANSLATION OF PLASMODIAL RNA: RNA isolated from plasmodia late in the G2 phase was

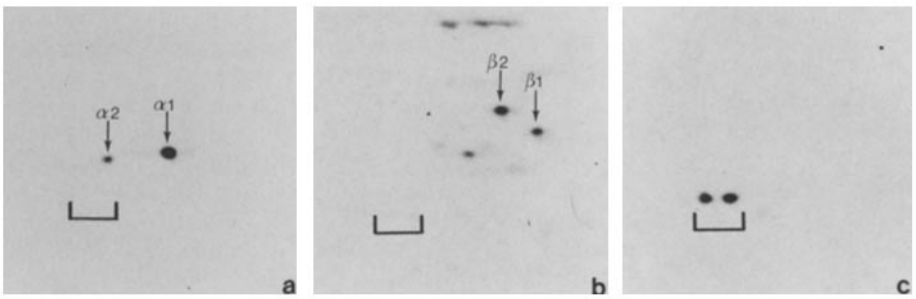

FIGURE 6 2D gel electrophoresis of proteins translated in vitro from hybrid-selected poly A^+ RNA. (*a*) Hybrid selection by a homologous α-tubulin DNA sequence. (*b*) Hybrid selection by heterologous β-tubulin DNA sequences. (*c*) Hybrid selection by a heterologous actin DNA sequence. See the legend to Fig. 1 for 2D gel conditions. The horizontal bar delineates the position of the actin in vitro translation products.

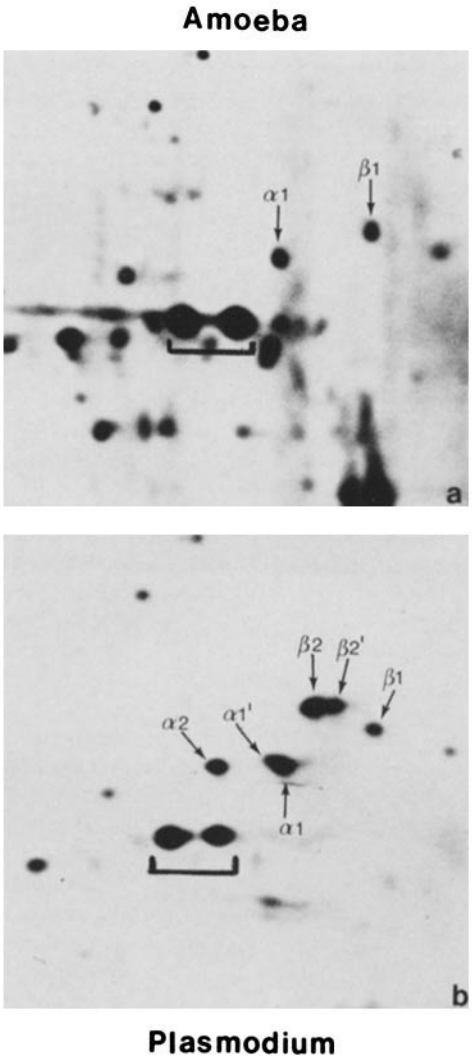

FIGURE 7 2D gel electrophoresis of proteins translated in vitro from total cellular RNA. (*a*) In vitro translation products of myxamoebal RNA. (*b*) In vitro translation products of plasmodial RNA. See Fig. 1 for 2D gel conditions. The horizontal bar delineates the position of the actin in vitro translation products.

translated in vitro, and the products were resolved in 2D gels. The α1-, α2-, β1-, and β2-tubulins observed among in vivo-labeled plasmodial proteins were also observed among the in vitro translation products (Fig. 7*b*). The identities of the tubulins was confirmed by peptide mapping (Fig. 4). Since α2- and β2-tubulins were not observed among the in vitro translation products of myxamoebal RNA, they cannot arise by posttranslational modifications of the α1- and β1-tubulins in the wheat germ system. Thus, the α2- and β2-tubulins are encoded by mRNA distinct from those encoding the α1- and β1-tubulins, respectively; therefore, there is probably at least one gene each for the α1-, α2-, β1-, and β2-tubulins. However, the possibility remains that the α2- and β2-tubulins are encoded by mRNA that arise by differential readouts of the α1- and β1-tubulin genes.

As with in vivo-labeled polypeptides, 2D gel electrophoresis of in vitro translation products indicated that the plasmodial α1- and β2-tubulins may be complex electrophoretic species (Fig. 7*b*). Peptide mapping of the α1′- and β2′-spot regions failed to detect any major presence of nontubulin proteins (Fig. 4).

DISCUSSION

Three α-tubulins and two β-tubulins have been resolved by 2D gel electrophoresis of *Physarum* polypeptides labeled in vivo. The identity of the tubulins was established by peptide mapping with *Staphylococcus* V8 protease and with chymotrypsin, using as primary standard myxamoebal tubulin purified by self-assembly into microtubules. Immunoprecipitation with a β-tubulin-specific monoclonal antibody independently established the identities of the β-tubulins. Translation in vitro of mRNA selected by hybridization to cloned tubulin DNA sequences further confirmed the identities of the α- and β-tubulins.

The *Physarum* tubulins showed different patterns of expression in the different cell types. The α1- and β1-tubulins were detected in both myxamoebae and plasmodia, although it should be noted that detection of α1- and β1-tubulin species in both myxamoebae and plasmodia does not necessarily mean that myxamoebal α1 and β1 are the same polypeptides as plasmodial α1 and β1. The α2- and β2-tubulins were detected only in plasmodia, and the α3-tubulin was detected only in the myxamoebal phase. The α3-tubulin was present only under conditions favoring development of myxamoebae into flagellates. The flagellar α-tubulins of both *Chlamydomonas* (21) and *Polytomella* (25) show different electrophoretic mobilities from the cytoplasmic α-tubulins. The relative positions on 2D gels of the *Chlamydomonas* cytoplasmic and flagellar α-tubulins (21) are remarkably similar to those of the α1- and α3-tubulins, respectively, of *Physarum* (Fig. 1), which tempts one to speculate that the *Physarum* α3-tubulin is specific to the flagellum.

The significance of the different patterns of expression of tubulins in *Physarum* is unclear. In the multitubulin hypothesis, Fulton and Simpson (11) proposed that different microtubular organelles may be assembled using different α- and β-tubulins, and thus that the differences in α- and β-tubulins have functional significance. Our observations suggest a specific function for α3-tubulin in the flagellum; however, only two α- and one β-tubulin species were detected in the myxamoebal phase, where cytoplasmic, mitotic-spindle, centriolar, and flagellar microtubules are all found (34). This would seem to indicate that different tubulins are not necessarily required to make some of the functionally different microtubules. A similar conclusion has also been recently deduced from genetic evidence in *Drosophila* (17). However, it is important to note that a single electrophoretic species may consist of more than one polypeptide species, so that the number of different tubulins detected in the myxamoebal phase should be regarded as a minimum.

It is remarkable that more species of tubulins were detected in plasmodia than in the myxamoebal phase, since growing plasmodia display microtubules only in the mitotic spindle (33). The complex shapes of the plasmodial α1- and β2-tubulin 2D gel spots suggest further that each may represent multiple tubulins. The possibility that some of the tubulins are not able to assemble into microtubules can be discounted, since all four plasmodial species are present not only among the tubulins purified by self-assembly in vitro of plasmodial extracts, but also among the proteins of the isolated mitotic apparatus (A. Roobol, personal communication). Whether the different α- and β-tubulins of the plasmodia have specialized functions or whether the differences simply reflect neutral drift of multiple gene products is not yet clear.

In vitro translation of total cellular RNA was used to

investigate whether the different α- and β-tubulins are the products of the same or different genes. The results indicated that different mRNA, and therefore probably different genes, encode the α1-, α2-, β1-, and β2-tubulins, although we cannot rule out the possibility that different RNA processing events could give rise to multiple products of one gene each for α- and β-tubulin. Thus, some of the differences in tubulin expression between myxamoebae and plasmodia may reflect differential gene expression.

The differential expression of tubulins that we have detected in myxamoebae and plasmodia will facilitate studies of the control of tubulin gene expression in different cell types of the same organism; the myxamoebae and plasmodia are different phases of the *Physarum* life cycle and can thus be cultured completely independently. The particular patterns of tubulin expression in the two phases provide an opportunity to elucidate whether multiple α- and β-tubulins reflect functional specializations or whether individual tubulin subunits are involved in multiple functions.

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