The Periodic Synthesis of Tubulin in the Physarum Cell Cycle

CHARACTERIZATION OF PHYSARUM TUBULINS BY AFFINITY FOR MONOCLONAL ANTIBODIES AND BY PEPTIDE MAPPING*

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Polypeptides preferentially labeled in the G2 phase of the synchronous nuclear replication cycle of Physarum macroplasmodia were compared in electrophoretic mobility and peptide map with the tubulins enriched from Physarum myxamoebae. One major and one minor fluorographic species matched the myxamoebal α and β chains, respectively. Thus, tubulins are among the proteins of Physarum selectively synthesized before nuclear division. A third species, Prominentlabeled in premitotic plasmodia, is distinct from the two myxamoebal tubulins even though it co-polymerizes with microtubules. The nature of P remains unknown.

Two rat monoclonal antibodies directed against yeast tubulin were found to bind selectively to the α tubulin of porcine brain. These served to confirm the assignment of the 50,000-dalton Physarum myxamoebal tubulin as an α-like polypeptide.

The natural synchrony of the syncytial macroplasmodium of Physarum polycephalum offers an opportunity to study the regulation of macromolecular synthesis in the nuclear division cycle (1). Previous work from our laboratory has shown that several polypeptides capable of co-polymerization with porcine brain microtubular protein are synthesized with pronounced periodicity (2). Where it has been measured, the rate of synthesis of these species is at least 30-fold greater before nuclear division than after. The molecular weights and isoelectric points of these periodic microtubular species resemble those of porcine brain tubulin in two-dimensional electropherograms.

Although it has not been possible to purify tubulin from Physarum plasmodia (3, 4), Gull and his colleagues (5, 6) have been able to purify by self-assembly two polypeptides from the myxamoebal form of Physarum. Defined by self-assembly, one myxamoebal tubulin has been found to be partially α-like and the other to be extensively β-like when compared to the mammalian tubulins (7).

In this report, we compare the periodic microtubular poly

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peptides of plasmodia with the α and β tubulins from Physarum myxamoebae, using as criteria mobility on two-dimensional electrophorograms, binding of monoclonal antitubulins (8), and peptide map by partial V8 proteolysis.

MATERIALS AND METHODS

Cultures—P. polycephalum myxamoebae (strain CLD-AXE (9)) were grown at 22 °C in a modified semidefined medium (10) in which 1% mycological peptone substituted for casein hydrolysate. Microplasmodia and plasmodia (strain CL (ATCC 24112)) were grown as described by Laffler et al. (2).

Electrophoresis—SDS-PAGE was carried out as described by Laemmli (11), utilizing technical grade SDS from Matheson, Coleman and Bell which allows separation of both porcine and Physarum tubulin subunits. Other reagents were from Bio-Rad. All slab gels were 1.5-mm thick. Molecular size markers, also from Bio-Rad, included phosphorylase b, 92,500; bovine serum albumin, 66,200; ovalbumin, 43,000; soybean trypsin inhibitor, 21,000; and lysozyme, 14,400. Gels were stained directly for protein with Coomassie brilliant blue (0.25% in 50% methanol, 10% acetic acid) and destained in 20% methanol with 1% acetic acid. Two-dimensional electrophoresis followed the O'Farrell procedure we have described (2), except that 0.4% SDS was included in the isoelectric focusing step (12). pH values were determined as described by O'Farrell (13) using degassed double-distilled water.

Protein Blot Transfer onto Nitrocellulose—After SDS-PAGE, the gel was equilibrated by gently shaking for 15-30 min in transfer buffer (50 mM NaCl, 2 mM Na-EDTA, 0.1 mM dithiothreitol, 10 mM Tris-HCl, pH 7.0) supplemented with 4 μg urea. The transfer of protein from the gels to nitrocellulose paper (BA 85, Schleicher and Schuell) was then accomplished by forming a sandwich with two sheets of nitrocellulose and allowing diffusion for 12-16 h in 2 liters of transfer buffer (14). Of the two protein blot transfers, one was normally used for protein staining and the other for testing antibody binding. Protein was stained with amido black (0.1% in 45% methanol, 10% acetic acid) for 30 min and then destained with 90% methanol and 2% acetic acid. Two-dimensional electrophoresis followed the O'Farrell procedure we have described (2), except that 0.4% SDS was included in the isoelectric focusing step (12). pH values were determined as described by O'Farrell (13) using degassed double-distilled water.

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-70 °C for 16 h unless specified otherwise.

**Vinblastine Precipitation of Physarum Protein**—Vinblastine precipitation was carried out as described by Kemphues et al. (16) modified as follows. One-tenth volume of vinblastine sulfate (Sigma) at 22 mg/ml was added to the clarified sonicate. After 1-2 h on ice, the precipitate was collected by centrifugation at 39,000 × g for 30 min at 4 °C. The pellet was dissolved in 1-2 ml of resuspension buffer (0.1 M 1,4-piperazinediethanesulfonic acid, pH 6.94, 1 mM ethylene glycol bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, 0.1 mM MgCl₂, 1% Trasylol) supplemented with 2 mM GTP. The protease inhibitor Trasylol was obtained from Mobay Chemical Corp., FBA Pharmaceuticals. The milky white supernatant was collected after brief centrifugation and stored in aliquots at −70 °C.

**Purification of Microtubular Protein from Porcine Brain and Physarum**—Porcine brain microtubular protein and purified brain tubulin (17) were kindly provided by Dr. Robert Scheele (University of Wisconsin). Physarum myxamoebal microtubular proteins were purified by self-assembly (18).

**Hybridoma Cell Lines and Antibodies**—Kilmartin and his colleagues (8) have prepared monoclonal antibodies to tubulin by fusing with rat myeloma cells the splenocytes of rats hyperimmunized with partially purified yeast tubulin. Among the antibodies that were positive for binding to yeast tubulin, a subset was also positive with chick brain tubulin. Two of these cross-reactive antibodies, YL1/2 ("2") and YOL1/34 ("34"), were unique in also staining the mitotic spindle of yeast and the interphase microtubules of Chinese hamster ovary and 3T3 cells, as judged by indirect immunofluorescence. Hybridoma 2 emerged from a fusion employing the rat myeloma line Y3/Ag1.2.3, secreting its own light chain. Both antibodies, 2 and 34, are of the IgG class (2). We confirmed that both antibodies were active in binding porcine tubulin using a solid phase microtiter well binding assay modified from that of Nowinski et al. (15). In addition, we also found that antibodies stained porcine microtubules polymerized in vitro by indirect immunofluorescence.

**Tubulin Peptide Map from Two-dimensional Gels**—A labeled plasmoidal extract was mixed with myxamoebal tubulins (the pellet fraction from the third cycle of warm assembly, H,P) and co-electrophoresed on two-dimensional gels. Regions of the gel corresponding to the periodic microtubular proteins were detected by fluorography and cut out. Partial proteolysis by Staphylococcus aureus V8 protease (19) was analyzed on 15% polyacrylamide gels. The gels were stained with Coomassie brilliant blue, treated with ENHANCE (New England Nuclear), dried, and exposed to Kodak X-AR-5 film for 42-48 days.

**RESULTS**

The timed synthesis of microtubular proteins in the Physarum plasmoidal can be demonstrated by radiolabeling polypeptides that are resolved by two-dimensional electrophoresis (2). When a surface plasmoidal was incubated with [35S]methionine for 2 h before nuclear division, the microtubular species were labeled maximally. The total plasmoidal extract was then analyzed on a twodimensional O'Farrell gel. A fluorogram of the region of interest is shown in Fig. 1. The G2-labeled plasmoidal proteins of the plasmodium lie in the molecular size range of 48,000-57,000 and the pI range of 5.08-5.56. The intense set of spots to the lower left of the electropherogram may be actin ("A"); apparent mass 42,500 daltons; apparent pI of 5.56). We have previously shown that co-polymerization with porcine brain tubulin enriches the periodic species but not the presumptive actin (2).

**Purification and Antigenic Characterization of Physarum Tubulins**—We tested whether the interspecies reactivity of rat monoclonal antibodies raised against yeast tubulins could be used to identify tubulin-related polypeptides in the myxomycete *P. polycephalum*. Tubulin from myxomycobae of Physarum was purified by self-assembly (6, 7) or enriched by vinblastine precipitation. Polypeptides were resolved by SDS-PAGE and a dot transfer was tested for reactivity to monoclonal antibulbin antibodies.

We found that antibody 2 strongly labeled the α subunit of porcine tubulin of molecular weight 56,500 (Fig. 2, lane b).

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**FIG. 1.** Periodic microtubular proteins in the plasmoidal of *P. polycephalum*. One-half of a plasmoidal was labeled with 200 μCi of [35S]methionine for 2 h before mitosis II (1). The total plasmoidal extract (2,000,000 dpm/gel) was resolved on a two-dimensional O'Farrell gel and detected by fluorography. Only the middle portion of the lower half of the gel is shown here. Regions are indicated that correspond to α tubulin, β tubulin, presumptive actin (A), and the periodic microtubular polypeptide P.

**FIG. 2.** Immunostaining of Physarum microtubular proteins by antibody 2. Self-assembled myxamoebal protein at H,P (15 μg) was mixed with molecular weight markers and run in lanes a and j. Porcine brain tubulin (4 μg) was run in lane b; 200 μg of vinblastine-fractionated amoebal proteins in lane d (precipitate fraction) and in lane e (supernatant fraction); and 200 μg of vinblastin-fractionated microplasmodial proteins in lane f (precipitate fraction) and in lane h (supernatant fraction). Vinblastine-treated microplasmodial material was further concentrated by acetone precipitation and run in lanes g and i. Lane g, 200 μg of vinblastine supernatant; lane h, 200 μg of vinblastin supernatant; and lane i, 400 μg of vinblastin precipitate. The top section is a nitrocellulose protein blot immunostained with anti-α tubulin antibody; the bottom section is a gel stained for total protein. Heavy arrows locate the polypeptide bands that bind anti-α antibody.
Antibody 34 exhibited the same reactivity as shown. The α-specific antibody mobility of the α-like polypeptide is similar to the 50,000-dalton band of the vinblastine-precipitated myxamoebal tubulin (Fig. 2, lane d). Notice that antibody 2 binding shows that the α-like Physarum tubulin has been enriched in the vinblastine precipitation compared with the supernatant (Fig. 2, lane d versus c), whereas Coomassie blue staining did not reveal such a clear distinction.

We also examined whether the tubulin of Physarum microplasmodia could be enriched by vinblastine precipitation on the basis of similar criteria. We did not detect any material stainable by Coomassie blue in the tubulin regions covering both porcine and Physarum amoebal tubulins (Fig. 2, lanes e–i). Antibody 2 also failed to identify any α-like tubulin in the microplasmodial material. Surface plasmodia were found also to lack polypeptides in the tubulin region (data not shown).

A minor reaction of antibody 2 can be seen in Fig. 2, lanes c, d, and i. This 57,000-dalton band is not enriched by vinblastine precipitation, is absent from self-assembled microtubules, and is not detected by antibody 34.

Comparison by Electrophoresis—Since plasmodia seem to contain very little tubulin, direct identification of the periodically labeled polypeptides is not feasible. Therefore, we were obliged to compare the electrophoretic mobilities of both myxamoebal and porcine tubulin with those of radionuclide-labeled periodic polypeptides. We found that the less acidic myxamoebal tubulin subunit binds anti-α antibody (Fig. 3). This α-like myxamoebal tubulin has an isoelectric point of 5.31; the second myxamoebal tubulin focuses at 5.08. As shown in Fig. 4, co-electrophoresis of labeled periodic polypeptides with myxamoebal tubulin revealed that two of the periodic species had electrophoretic mobilities similar to myxamoebal tubulins. The species that co-migrate with the myxamoebal β chain is minor in the fluorogram. However, in Fig. 5B, it can be seen as a prominent spot that co-migrates with the porcine β chain. This spot is enriched by co-polymerization. We could not, however, demonstrate identity of porcine α tubulin to any of the periodic plasmodial polypeptides even after co-polymerization.

The major periodic polypeptide designated “P” by us (2) has an isoelectric point intermediate between those of α and β myxamoebal tubulins. It was also enriched after co-polymerization (Fig. 5B).

Comparison by Peptide Mapping—Further characterization of the periodic plasmodial molecules was achieved by peptide mapping after limited proteolysis (19). In Fig. 6A, myxamoebal tubulin is detected by protein staining, while the labeled plasmodial polypeptides from the co-electrophrogram depicted in Fig. 4 are detected by fluorography. The quality of these peptide maps is limited by the very small amounts of material eluted from these two-dimensional gels. Myxamoebal α tubulin (lane b) shows clear similarity to the periodic polypeptide with which it co-migrates (lane a). Myxamoebal β tubulin (lane d) shows peptide map similarity...
from plasmodia and were resolved as described in the legend to Fig. 4. The plasmodial species were developed after 42-48 days of exposure. Myxamoebal tubulin bands were eluted from a preparative SDS-PAGE gel and a fluorogram of labeled plasmodial peptides that co-migrate with mammalian tubulin. We have extended the observations of Kilmartin and his colleagues in showing interspecies reactivity to porcine brain tubulin and to Physarum myxamoebal tubulin. Additionally, we have found that each antibody is selective for the α subunit of purified porcine brain tubulin and for the 50,000-dalton polypeptide purified from Physarum myxamoebae by self-assembly or by vinblastine precipitation. Thus, each antibody fulfills the necessary conditions for a reagent to detect the α tubulin chain. It is plausible that the monoclonal antibody recognizes a very small portion of the peptide either in porcine or Physarum α tubulin and thus permits positive and specific identification of this subunit even when neither the peptide map nor the SDS-PAGE mobility is conserved. These specific antibodies will provide a useful tool in defining the α subunit in a broad range of species; this circumvents the difficulty experienced in identifying α tubulin on the basis of mobility in SDS-PAGE (7, 20).

Despite variation in electrophoretic mobility of the Physarum myxamoebal α tubulin, conservation in peptide mapping has been inferred by Little et al. (29) and recently confirmed by Roobol and Gull (21) using chymotrypsin rather than V8 protease as the proteolytic agent.

How are the periodically labeled plasmoidal molecules of Physarum related to tubulin? Our previous labeling study showed that there is a strong periodicity in synthesis of polypeptides in the tubulin region (2). However, the characterization of plasmoidal tubulin is limited by its low level even after vinblastine enrichment. To resolve this difficulty, we compared labeled periodic polypeptides with both myxamoebal and porcine tubulin. We have concluded that plasmoidal β tubulin is similar to each reference type of β tubulin in two-dimensional electrophoretic mobility. This plasmoidal species gives only a faint fluorographic signal and has not been quantified previously (2). Judged visually, this minor β-like species seems to be periodic in its synthesis. This species is also highly enriched after two cycles of co-polymerization with porcine tubulin (Fig. 5).

The set of periodic polypeptides denoted P includes two major and one minor fluorographic species. They fail to correspond either to self-assembled myxamoebal tubulin or to porcine tubulin in electrophoretic properties and in peptide map. Yet set P efficiently co-polymerizes with carrier porcine tubulin (2) (Fig. 5). P may be either a plasmodium-specific tubulin or a microtubule-associated protein. Further study of co-polymerization of P with carrier myxamoebal tubulin may yield useful information as to the identity of P.

**DISCUSSION**

A key element in our characterization of Physarum tubulins is the use of the broadly reactive rat monoclonal antibodies of Kilmartin et al. (8). We have extended the observations of Kilmartin and his colleagues in showing interspecies reactivity to porcine brain tubulin and to Physarum myxamoebal tubulin. Additionally, we have found that each antibody is selective for the α subunit of purified porcine brain tubulin and for the 50,000-dalton polypeptide purified from Physarum myxamoebae by self-assembly or by vinblastine precipitation. Thus, each antibody fulfills the necessary conditions for a reagent to detect the α tubulin chain. It is plausible that the monoclonal antibody recognizes a very small portion of the peptide either in porcine or Physarum α tubulin and thus permits positive and specific identification of this subunit even when neither the peptide map nor the SDS-PAGE mobility is conserved. These specific antibodies will provide a useful tool in defining the α subunit in a broad range of species; this circumvents the difficulty experienced in identifying α tubulin on the basis of mobility in SDS-PAGE (7, 20).

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![Fig. 6. Peptide mapping of periodic microtubular proteins from plasmodia and of tubulin from myxamoebae of Physarum and porcine brain. A, mixtures of trace amounts of [35S]methionine-labeled plasmoidal polypeptides and unlabeled myxamoebal tubulins were resolved as described in the legend to Fig. 4. The plasmoidal peptides were detected by fluorography, and the corresponding regions of the gels were cut out and subjected to proteolysis by S. aureus V8 protease. Fluorograms showing the peptide maps of plasmoidal species were developed after 42-48 days of exposure. Lane a, fluorogram of labeled plasmoidal peptides that co-migrate with myxamoebal α tubulin; lane b, Coomassie blue staining pattern of peptides derived from myxamoebal α tubulin; lane c, fluorogram of the peptides derived from the labeled plasmoidal polypeptides in the myxamoebal β tubulin region; lane d, Coomassie blue staining pattern of myxamoebal β tubulin; lane e, fluorogram of the labeled peptides derived from the major plasmoidal microtubular polypeptide P. B, tubulin bands were eluted from a preparative SDS-PAGE gel and then digested with V8 protease. Proteolytic fragments were resolved to the periodic plasmoidal polypeptide with which it co-migrates (lane c). The similarity of myxamoebal β tubulin to mammalian β tubulin is seen in Fig. 6B (lane d versus c). Some differences in myxamoebal α tubulin are seen in Fig. 6B, lane a. Finally, the prominent periodic species P (Fig. 6A, lane e) resembles neither myxamoebal tubulin in peptide map. This unique cleavage pattern was seen over a range of digestion conditions for labeled material eluted from the P region of Fig. 4. It is unaffected by the inclusion of carrier α or β tubulin from porcine brain or from Physarum; it seems to be an intrinsic and distinctive property of this set of radiolabeled species.](image-url)
Physarum Tubulins

Normally, the $\alpha$ and $\beta$ tubulin subunits function as a heterodimer and would be found in stoichiometrically equivalent amounts. However, the $\beta$ subunit that we have identified in the set of periodically synthesized polypeptides is apparently less abundant than the identified $\alpha$ subunit. Alternatively, this subunit is methionine-poor. Further work, including resolution of the nature of $\beta$, will be required to understand the significance of this apparent lack of equivalence.

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