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# Synthesis and Some Properties of an Actin-Like Nuclear Protein in the Slime Mold *Physarum polycephalum*

BRIGITTE M. JOCKUSCH, DONNA F. BROWN, AND HAROLD P. RUSCH

*Max-Planck-Institut für Biologie, Abteilung Melchers, D 74 Tübingen, West Germany, and McArdle Laboratory for Cancer Research, Medical Center, University of Wisconsin, Madison Wisconsin 53706*

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A protein was extracted from isolated nuclei of the slime mold *Physarum polycephalum* which could be labeled with radioactive precursors only during $G_2$ phase. The native protein was purified by extraction in low-ionic-strength buffer [10 mM tris(hydroxymethyl)aminomethane-hydrochloride] of isolated nuclei and by preparative polyacrylamide gel electrophoresis. It was extracted from isolated nucleoli. Its electrophoretic properties in three different polyacrylamide gel systems, its molecular weight (44,000 ± 3,000), its precipitability by vincaleucoblastine, a vinca alkaloid, and its aggregation properties suggested that it might be actin. In a direct comparison with slime mold actin purified from the cytoplasm, no difference could be found between the two proteins in all these characteristics. The synthesis of cytoplasmic actin was not found to occur exclusively during $G_2$ phase. This suggested that nuclear actin was either synthesized independently from cytoplasmic actin or transported into the nuclei exclusively during $G_2$ phase. The possible role of nuclear actin during intranuclear mitosis is discussed.

In eukaryotes, few organisms may provide as good a material for studying nuclear proteins as the slime mold *Physarum polycephalum*. The mold can be grown as a syncytium in large quantities (18), in fully defined or semidefined medium (6). When plasmodia are kept in the dark at 27 C, nuclei will undergo a synchronous mitotic division every 8 hr. These mitotic divisions are intranuclear, e.g., the nuclear membranes remain more or less intact throughout the whole process (10, 13, 21). This feature allows the isolation of nuclei in all stages of the mitotic cycle (J. Mohberg and H. P. Rusch, Exp. Cell Res., *in press*). Nuclear proteins may be extracted from isolated nuclei, and their synthesis throughout the mitotic cycle may be studied by labeling the plasmodia with radioactive amino acids before the isolation of nuclei.

Among the nuclear proteins, we were especially interested in structural proteins and their possible role during mitosis. In organisms where nuclear membranes disintegrate at the beginning of mitosis, it is likely that the material required for construction of the mitotic apparatus is provided by the cytoplasm, but an intranuclear spindle may demand that the proteins of the mitotic apparatus be present inside the nucleus before the onset of mitosis. In a previous report (15), we demonstrated a nuclear protein that was labeled with $^{14}$C-amino acids exclusively during the $G_2$ phase of the mitotic cycle (*Physarum* lacks a $G_1$ period in its plasmodial stage), whereas histones were labeled during S-phase (20). In the present work, we further purified this "protein X" and identified it as a structural protein, actin, and studied its synthesis during the mitotic cycle to determine whether the phase-specific labeling reflects phase-specific synthesis or transport into the nuclei.

## MATERIALS AND METHODS

**Culture of the organism.** *P. polycephalum* strain $M_3C$ (6), subline $M_3C$ V, was grown in axenic culture in shake flasks at 22 to 23 C (18). Surface plasmodia were formed by coalescence of microplasmodia from 1-day-old shake flasks (middle of log-phase growth) on membranes (Millipore Corp., Bedford, Mass., HAWP 0010, or Schleicher und Schüll, Dassel, Germany, cellulose nitrate filters "Selektronfilter," BA 85/0), either in petri dishes (small plasmodia) or in stainless steel pans (large plasmodia) on rocker platforms (18). The small plasmodia were incubated at 27 C, and the large ones were kept at room temperature. Mitosis was determined by phase-contrast microscopy of biopsies fixed in ethanol (12). At 27 C, the mitotic cycle lasted about 8 hr.

**Isolation of nuclei and nucleoli.** Nuclei were isolated from fresh plasmodia at different times during the cell

cycle, by homogenizing the mold in an isolation medium containing Triton X-100 and pelleting the nuclei by centrifugation through 1 M sucrose (J. Mohberg and H. P. Rusch, Exp. Cell Res., *in press*). After the nuclei had been spun twice through 1 M sucrose, they were free from visible contamination, as judged by phase-contrast microscopic examination. Nucleoli were isolated directly from plasmodia by the following procedure (J. Mohberg and H. P. Rusch, Exp. Cell Res., *in press*). Plasmodia were homogenized in the same medium as was used for isolation of nuclei, but with calcium or magnesium content lowered to one-tenth of that used for nuclear isolation. In this medium, nuclear membranes were disrupted during homogenization, and the freed nucleoli could be further purified in the same way as whole nuclei.

**Extraction of cytoplasmic, nuclear, and nucleolar proteins.** Cytoplasmic extracts (100*S*) were prepared by homogenizing pieces of plasmodia, removing the nuclei by low-speed centrifugation (Sorvall Superspeed RC 2-B or MSE High Speed 18, 500 × *g*, 4 C, 15 min), and pelleting the insoluble particles of the cytoplasm by centrifugation (Spinco, model L2, 100,000 × *g*, 4 C, 1 hr). Isolated nuclei and nucleoli (either fresh or frozen) were extracted by sonic treatment (Branson Sonifier, models LS 75 or 125; regular microtip: setting 4, 1 to 2 min; special microtip: setting 2 or 3, 2 min), either in 67% acetic acid (9) or in an aqueous buffer. The buffer systems used varied and were either tris(hydroxymethyl)aminomethane-hydrochloride (Tris) buffer, *p*H 7.3, in concentrations varying from 10 mM to 1 M, or 0.01 M potassium phosphate plus 0.0015 M $MgCl_2$, *p*H 8.3. Cleland reagent or 2-aminoethanethiol hydrochloride was added to all extraction media for SH-group protection, in concentrations of 1 to 5 mM. When plasmodia or nuclei had been disrupted by sonic treatment, microscopic examination revealed no intact nuclei after 1 or 2 min. Isolated nucleoli, however, had not completely disintegrated but seemed somewhat less compact than untreated nucleoli. After sonic treatment, the extracts were centrifuged at 20,000 × *g* (Sorvall Superspeed RC 2-B, or MSE High Speed 18, 4 C) for 15 min. The residues were either reextracted with 67% acetic acid, to solubilize buffer-insoluble proteins (see Fig. 1), or discarded. The supernatant fractions were

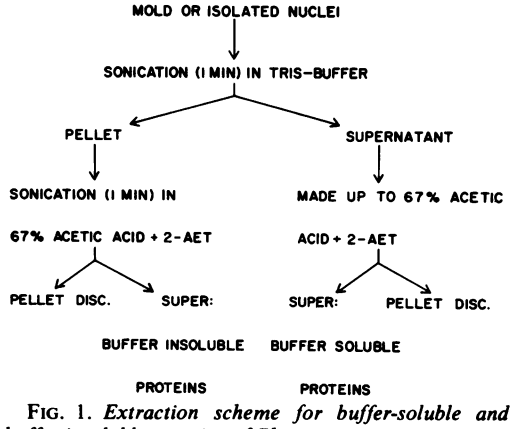


FIG. 1. *Extraction scheme for buffer-soluble and buffer-insoluble proteins of Physarum.*

usually dialyzed overnight at 4 C against the same buffer used for extraction. Samples were used for protein determination by the method of Lowry et al. (17), with bovine serum albumin as a standard, and carbohydrate determination by the phenol sulfuric acid method, with glucose as a standard (7).

**Precipitation of proteins with vincaleucoblastine.** Extracts of slime mold cytoplasm, nuclei, or nucleoli in phosphate-magnesium buffer, *p*H 8.3, at a protein concentration of 1 to 2 mg/ml, were made $5 \times 10^{-3}$ M in vincaleucoblastine (VELBAN, vinblastine sulfate, Eli Lilly & Co., Indianapolis, Ind.) for precipitation of acidic proteins. They were allowed to stand at room temperature for at least 30 min. After centrifugation (15,000 × *g*, Sorvall Superspeed RC 2-B or MSE High Speed 18, 22 C, 10 min), the pellets were suspended in 0.2 ml of 0.01 M phosphate buffer and dialyzed against the same buffer until the samples were completely clear (8 to 24 hr, several changes of buffer).

**Radioactive labeling of proteins and determination of radioactivity.** Labeling of cultures was essentially the same as described previously (15). For experiments with radioactively labeled proteins, only small plasmodia were used. At different times during the cell cycle, plasmodia were transferred for 2.5 hr onto new petri dishes containing 5 μCi of $^{14}$C-lysine (315 Ci/mole) plus 5 μCi of $^{14}$C-leucine (338 Ci/mole, both from Schwarz BioResearch, Inc., Orangeburg, N.Y.) or $^{14}$C-protein hydrolysate (52 Ci per equivalent carbon atom, The Radiochemical Centre, Amersham, England) per ml of regular growth medium. At the end of the labeling period, plasmodia were rinsed in ice cold tap water and homogenized. Nuclear or cytoplasmic proteins were dialyzed against buffer or precipitated with acetone, washed, and redissolved in 1% sodium dodecyl sulfate (SDS) plus 8 M urea and 5 mM Cleland Reagent at 50 C. Radioactivity was determined with a Packard Tri-Carb or a Beckman LS230 scintillation counter by mixing samples with 10 ml of scintillation fluid ["ANPO," containing 2,5-diphenyloxazole (PPO), α-naphthylphenyloxazole, dioxane, xylene, and ethanol; or Scintisol, Packard, Inc.; or toluene-ethylene glycol, 1:1, plus PPO and 1,4-bis-2-(5-phenyloxazolyl)-benzene]. To determine radioactivity of fractions of polyacrylamide gels, gels were forced through a fine wire gauze in a hand-driven gel-crushing device which yielded about 10 fractions per cm of gel. These were incubated overnight at 60 to 70 C in 0.4 ml of distilled water or 1% SDS and mixed with scintillation fluid; readioactivity was measured in a scintillation counter, as described above. The counting efficiency for gel fractions was 50 to 60%.

**Gel electrophoresis.** For separating proteins by electrophoresis, three systems of polyacrylamide gels were used. For electrophoresis of native proteins, gels were made with 5% polyacrylamide (including 0.3% bisacrylamide), and Tris-glycine buffer (*p*H 8.3) was used as electrolyte (25). The proteins were dissolved in phosphate-magnesium buffer. In this system, almost all proteins migrate as anions. This type of gel electrophoresis was also used to isolate the "front-running proteins." Gels were made 50 mm long, and a free space was left at the bottom of the gel tube which was filled with electrophoresis buffer and sealed by a piece of dialysis membrane fixed tightly over the lower end of the gel

tube. These gels were run for 17 to 20 hr at 60 v, at which time the bromophenol blue marker indicated that the front had moved into the space between the gel and the membrane. The proteins migrating with the front were removed with a syringe and were thus recovered from the gels.

The second type of gel electrophoresis was used mainly for determination of molecular weights of polypeptides (22). Gels were made 7.5% in polyacrylamide (including 0.5% bisacrylamide) and contained 0.1% SDS plus 8 M urea in 0.1 M Tris buffer, *p*H 7.3. Slime mold proteins in phosphate or Tris buffer, or 67% acetic acid, were precipitated with acetone (75% final concentration) and redissolved in 1% SDS, 8 M urea, 0.1 M Tris buffer plus 0.5% Cleland Reagent, at 50 C (15, 16). Complexed with SDS, all proteins run as anions. Molecular weights of slime mold proteins were calculated by co-electrophoresing them with the following marker proteins: human transferrin, molecular weight 72,000; bovine serum albumin, molecular weight 66,000; deoxyribonuclease I (bovine pancreas), molecular weight 31,000; elastase (bovine pancreas), molecular weight 25,000; bromegrass mosaic virus (coat protein subunit), molecular weight 20,000; tobacco mosaic virus (coat protein subunit), molecular weight 17,550; and bovine hemoglobin (subunit), molecular weight 17,000. Purified slime mold actin, which was also used for comparison of molecular weight, was a gift from S. Hatano, Nagoya University, Japan; isolated slime mold histones were provided by J. Mohberg. Before electrophoresis, marker proteins and slime mold proteins were adjusted to 50 to 200 $\mu$g of protein by the method of Lowry et al. (17).

To run proteins as cations, a third type of electrophoresis was used. Gels were 7.5% in polyacrylamide (including 0.5% bisacrylamide) and contained 8.8% formic acid plus 8 M urea (25). The electrolyte was 8.8% formic acid plus 8 M urea and 0.5% 2-aminoethanethiol hydrochloride as SH-group protector. Protein extracts in phosphate or Tris buffer, or vincaleucoblastine precipitates, were dialyzed against the same buffer before electrophoresis. Crystal violet was added as a marker dye.

To compare positions or patterns of proteins in the acidic or the SDS gels, split gels were used in many experiments. By inserting a paraffin-coated paper strip into the uppermost 5 mm of a gel and heating the tube, the upper end of a gel tube was divided into two compartments (E. Kaltschmidt and I. Hindennach, *unpublished data*). Two samples could thus be applied and run on the same gel without being mixed. In all three systems, electrophoresis was carried out in a Canalco electrophoresis apparatus, or in a locally made electrophoresis machine (MPI für Biologie, Tübingen, Germany, machine shop). After electrophoresis, gels were fixed, stained, and destained as described previously (15). Densitometer tracings were taken with a Canalco microdensitometer (model E) connected to a Sargent recorder (SRL) or with a Joyce-Loebl Chromoscan. In the case of split gels, photographs were taken with a Leica camera.

**Aggregation experiments.** To test the ability of slime mold proteins to aggregate, nuclei were extracted by sonic treatment in 10 mM Tris buffer, *p*H 8.0, containing 1 mM adenosine triphosphate (ATP) and 1 mM Cleland Reagent (1). From the 20,000 × *g* supernatant fraction (Sorvall Superspeed RC 2-B or MSE High Speed 18, 4 C, 15 min) of this extract, a sample was dialyzed against 0.01 M Tris-maleate buffer, plus 0.15 M KCl, 1 mM ATP, and 5 mM Cleland Reagent, *p*H 6.1 (1). In both cases, dialysis time was 3 to 4 hr. The sedimentation pattern of both samples was obtained by centrifugation in an analytical ultracentrifuge (Spinco model E, 50,740 rev/min, 20 C, Schlieren optics).

## RESULTS

**Separation of protein X from other nuclear proteins.** To study the properties of protein X, separation from the other nuclear proteins was essential. Therefore, we divided the proteins of isolated nuclei into two fractions (Fig. 1): those which could be easily extracted by aqueous buffers and those which were solubilized only by acetic acid extraction (9, 16). Protein X was identified by comparing its characteristics with previous studies on this protein (15). It was easily buffer-soluble, as revealed by subsequent electrophoresis of the extracts of buffer-soluble and total nuclear proteins (Fig. 2). In the pattern of total nuclear proteins, the proteins migrating the farthest could be identified as histones by comparing them with the pattern of isolated histones (19). Extraction of isolated nuclei in an aqueous buffer of very low ionic strength (10 mM Tris buffer), at almost neutral *p*H (7.3), yielded a solution of almost exclusively protein X, provided that an SH-group protecting agent was present and the sample was heated during treatment with SDS-urea. With increasing ionic strength of the Tris buffer, more and more different nuclear proteins appeared on SDS-urea gels (Fig. 3), among which were the histones (4).

**Labeling characteristics of buffer-soluble and -insoluble nuclear proteins.** Buffer-soluble and buffer-insoluble proteins, derived from nuclei isolated from plasmodia which had been labeled in S and $G_2$ phase, respectively, were separated on SDS-urea gels (Fig. 4). Our preliminary result (15), which demonstrated that protein X was labeled in $G_2$ phase only, was verified. The separation of total nuclear proteins into buffer-soluble and -insoluble fractions gave simpler radioactivity patterns than was the case with total nuclear extracts. Protein X was extracted exclusively in aqueous buffer and labeled only in $G_2$ phase, but the histones appeared only in the buffer-insoluble fraction of the nuclear proteins and were labeled only during S-phase (20).

**Localization of protein X inside the nucleolus.** In an attempt to localize the protein X, isolated nucleoli which had been proven free of intact nuclei in a microscopic examination were extracted by sonic treatment in acetic acid. The extracts of nucleolar proteins thus obtained re-

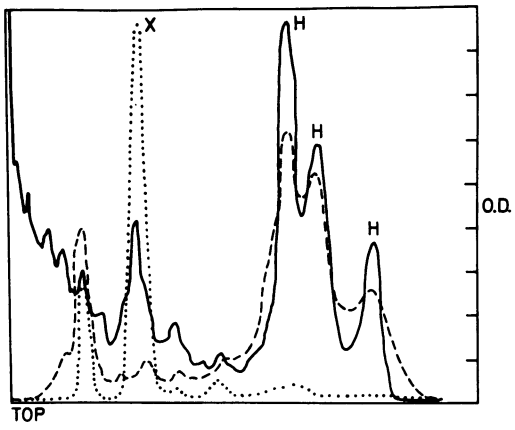


FIG. 2. *Densitometer tracings of stained patterns of nuclear proteins in SDS-urea gels. Solid line, total nuclear proteins, extracted sonically with 67% acetic acid and precipitated with acetone; dashed line, isolated slime mold histones; dotted line, extract of nuclear proteins in 10 mM Tris-hydrochloride plus 1 mM Cleland reagent, pH 7.3, precipitated with acetone. All three samples were solubilized in 1% SDS, 8 M urea, 0.5% Cleland reagent in 0.1 M Tris-hydrochloride, pH 7.3, before electrophoresis. Gels were 80 by 6 mm and contained 7.5% polyacrylamide in 0.1 M Tris-hydrochloride, pH 7.3, plus 0.1% SDS and 8 M urea. Gels were run for 4 hr at 60 v. Polypeptides complexed with SDS migrated as anions and were stained with Amido Black 10 B after the run (15). Ordinate, optical density (OD) in relative units; abscissa, the first 65 mm of the gel. H, histone bands; X, protein X. One band in the histone extract moved to the same position as the minor band in the aqueous nuclear extract. This is apparently a coincidence, because in determination of all other characteristics (e.g., migration in other gel systems, solubility, extractability), histone behaved quite differently. The amount of the minor band in the aqueous extract varied considerably.*

vealed, on SDS-urea gels (Fig. 5), a number of stainable bands and much material on top of the gels. We found protein X in nucleoli in large amounts as compared with the amounts of all other nucleolar proteins. The possibility that this result was due to an artifact may be minimized by the following. (i) The amount of this protein, represented by the area of the relevant peak in the densitometer tracing of the gel, did not decrease with the number of washings of nucleoli prior to extraction. (ii) Nucleoli contain about 60% of the total protein of the nucleus (J. Mohberg and H. P. Rusch, Exp. Cell Res., *in press*). In the densitometer tracing, protein X represents about 13% of the total nuclear protein (Fig. 5, solid line). Calculated from the tracing of nuclear proteins only, protein X should represent, therefore, 13/0.6 = 22% of the nucleolar protein, which is reasonably close to the 27% found by quantitative densitometry of the nucleolar pattern (Fig. 5, dashed line).

**Electrophoretic properties of the native protein X.** When extracts of nuclei or nucleoli in slightly alkaline aqueous buffer (phosphate or Tris buffer of low ionic strength), which contained almost exclusively protein X (Fig. 3), were electrophoresed in 5% polyacrylamide gels, buffered with Tris-glycine, without urea or SDS (25) ("glycine-buffered gels"), only one band was found after an anodic run, and this band coincided with the front marked by bromophenol blue. This method could therefore be used to purify protein X in any aqueous buffer, regardless of the ionic strength. Electrophoresis in glycine-buffered gels was continued until the front had left the gels and was rerun on SDS-urea gels. The proteins which migrated with the front in the glycine-buffered gel system revealed the same two-band pattern that appeared in the extract of nuclei in 0.01 M Tris buffer when separated directly on SDS-urea gels (Fig. 3, dotted line). The same result was found for nucleolar extracts. Also, the isolation of the fraction of a nuclear or nucleolar extract which migrated with the front in a glycine-buffered gel system was effective for separating these proteins from contaminating nucleic acids and polysaccharides, as shown by determination of carbohydrate content and optical density at 260 nm.

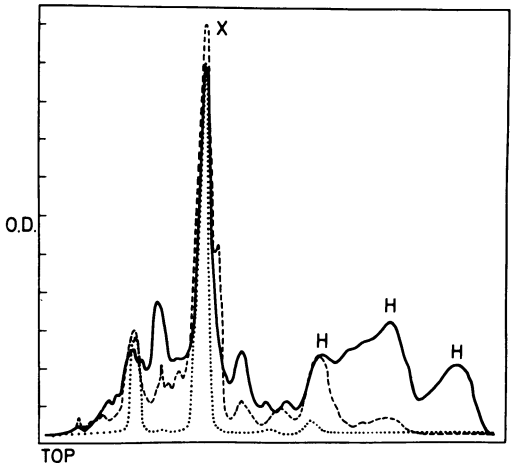


FIG. 3. *Densitometer tracings of stained patterns of nuclear proteins, extracted from isolated nuclei in 1 M (solid line), 0.1 M (dashed line), and 0.01 M (dotted line) Tris buffer, pH 7.3, plus 5 mM Cleland Reagent. In all three extracts, polysaccharides were precipitated with 67% acetic acid and proteins were denatured with 75% acetone before being dissolved in SDS-urea buffer. Gels and electrophoresis conditions as in Fig. 2. H, histone bands; X, protein X.*

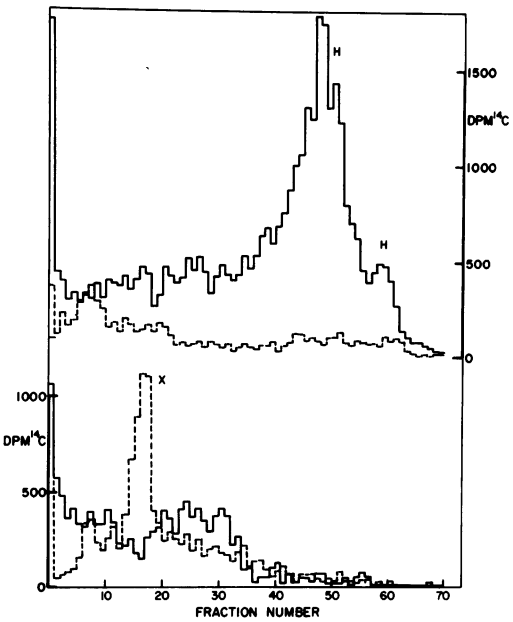


FIG. 4. *Radioactivity patterns of labeled nuclear proteins in SDS-urea gels. Nuclei were isolated from plasmodia which had been labeled in S- or late $G_2$-phase, with $^{14}$C-amino acids, for 2.5 hr. Nuclear proteins were extracted as in Fig. 1. Buffer-soluble and buffer-insoluble proteins were precipitated with acetone. Further treatment of proteins before electrophoresis, gels, and electrophoresis as in Fig. 2. Gels were fractionated after the run, radioactive proteins were eluted from the fractions, and radioactivity was determined in a scintillation counter. Upper panel: nuclear proteins extracted from plasmodia labeled in S phase; solid line, buffer-insoluble proteins; dashed line, buffer-soluble proteins. Lower panel: Nuclear proteins from plasmodia labeled in $G_2$-phase; solid line, buffer-insoluble proteins; broken line, buffer-soluble proteins. H, histones; X, protein X.*

**Molecular-weight determination of protein X.** From the fact that some nuclear proteins migrated with the front in the glycine-buffered system ("front-running proteins"), we concluded that they were very acidic or of small molecular weight, or both. To determine the molecular weights of the polypeptide chains, we coelectrophoresed marker proteins with nuclear front-running proteins in an SDS-urea gel system (22). The molecular weight of the large band (Fig. 3, dotted line) was found to be about 44,000 ± 3,000 (Fig. 6). (In preliminary experiments we had found 42,000; see reference 15). Thus, protein X is not very small, but of medium molecular weight. Therefore, its behavior in the glycine-buffered gel indicated that it is a very acidic protein. The small band in the upper part of the SDS-urea gel (Fig. 3, dotted line) is probably the dimer of the large polypeptide, for the following reasons. (i) It was present in all extracts, where otherwise only protein X was found, for example, in the front-running fraction of nuclear proteins, extract of low-ionic-strength buffer, and vincaleucoblastine precipitate (*see below*). (ii) The amount of this auxiliary band was rather high (up to one-third of protein X) when the sample was not heated properly during treatment with SDS and urea, or when the concentration of the SH-group protecting agent was lower than 1 mM. Furthermore, fresh samples contained very little of this material when prepared properly, but the amount of the auxiliary band increased with aging while the amount of protein X decreased, as could be seen in electrophoretic patterns on SDS-urea gels. (iii) The second band had a molecular weight of about 90,000, approximately 2 × 44,000, the molecular weight of protein X.

**Precipitation by vincaleucoblastine.** Since protein X resembled the structural proteins, microtubular protein and actin, in that it was acidic and it tended to form dimers, it was of interest to determine whether it would also complex with vincaleucoblastine (2, 3, 26). Therefore, we treated the front-running proteins, as well as total nuclear and total nucleolar extracts, with this alkaloid. A precipitate was formed at room temperature which could be solubilized by dialysis against aqueous buffers or acids (acetic or formic acid). When the resulting solution was examined on SDS-urea gels, the same two-band pattern (Fig. 3, dotted line) was found.

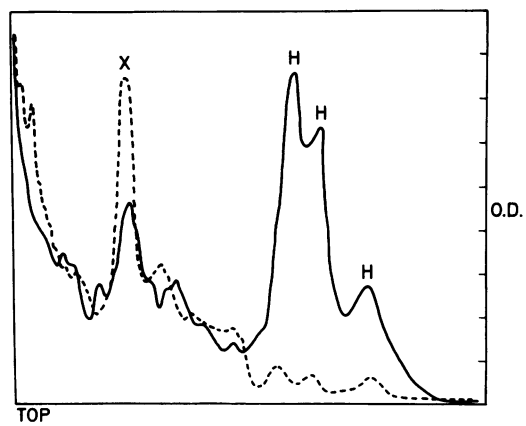


FIG. 5. *Densitometer tracings of stained patterns of total nuclear (solid line) and nucleolar (dashed line) proteins in SDS-urea gels. Nucleoli and nuclei were sonically extracted in 67% acetic acid; proteins were precipitated with acetone and solubilized in SDS-urea-containing Tris buffer. For gels and electrophoresis conditions, see Fig. 2. The same amount of protein (200 μg/ml) was layered on each gel. H, histone bands; X, protein X.*

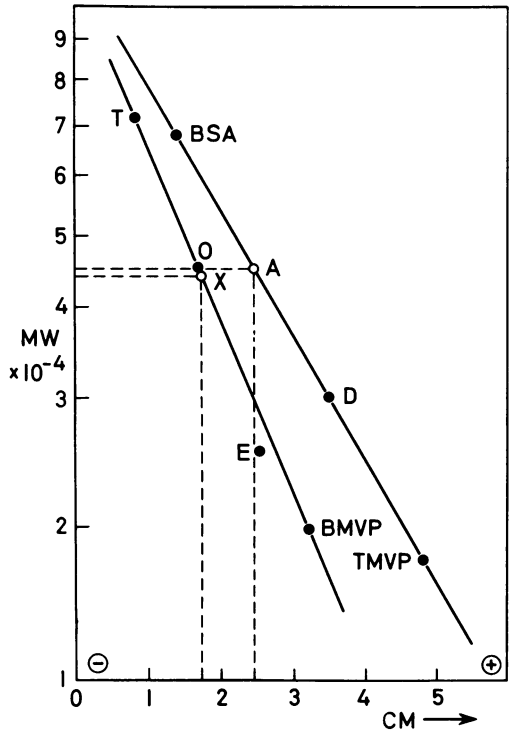


FIG. 6. *Molecular-weight determinations of nuclear actin-like protein X and purified cytoplasmic actin in SDS-urea gels (22). All proteins were in SDS-urea-containing Tris buffer. For gels and electrophoresis conditions, see Fig. 2. Two different experiments are shown in this graph. T, human transferrin; O, ovalbumin; E, elastase; BMVP, bromegrass mosaic virus coat protein; X, position of nuclear protein X. From this graph, a molecular weight of approximately 44,000 was determined. BSA, bovine serum albumin; D, deoxyribonuclease I; TMVP, tobacco mosaic virus coat protein; A, position of cytoplasmic actin, the molecular weight of which was determined as 45,000 in this experiment.*

**Comparison with purified slime mold actin.** Since the data presented above indicate that protein X might be either microtubular protein or actin, and since preliminary studies indicated that the molecular weight was more like that of actin (11), we compared the electrophoretic behavior of protein X with actin purified from the cytoplasm of *Physarum* (14). With the split-gel technique, we found that purified actin migrated in the 5% glycine-buffered gel with the front, as did protein X, and, in the 7.5% SDS-urea gel, it was found in the same position as the major band (Fig. 3) of the nuclear extracts. A molecular-weight determination of cytoplasmic actin of *Physarum* on SDS-urea gels is given in Fig. 6. There is good agreement between the molecular weights of protein X and cytoplasmic actin. However, there was still a slight possibility that the two proteins were not identical, since the front-running bands in the glycine-buffered system are not subject to separation, and molecular weights of polypeptides complexed with SDS were perhaps coincidental. Therefore we co-electrophoresed purified actin and purified protein X in a third gel system using again the split-gel technique. We chose an acid system (formic acid-urea), where even very acidic proteins should migrate as cations, thus testing the migration characteristics of both proteins determined by the positively charged groups. Again, the positions of the two proteins were identical (Fig. 7).

Both showed a main broad peak (probably comprising two bands) and a number of minor bands, in variable amounts. The reason for the appearance of more than one band in this gel system may be an enhanced formation of higher aggregates (dimers, trimers, etc.), because of the lack of many charged groups in the subunit. No acidic groups should be charged at this *p*H.

**Preliminary aggregation experiments with protein X.** Slime mold actin is known to form higher aggregates (F-actin) out of monomeric subunits (G-actin) when the conditions are changed from low-ionic-strength buffer, high *p*H, to high-ionic-strength buffer, low *p*H (1, 14), in a manner similar to rabbit actin. Therefore, we studied the aggregation properties of the actin-like nuclear protein, by using an analytical ultracentrifuge with schlieren optics. Extracts of nuclei in 10 mM Tris buffer were used, which contained almost no proteins except for the actin-like protein, as had been shown before by SDS-urea gel electrophoresis (Fig. 3). From these extracts (protein concentration, 1.5 mg/ml) a sample was dialyzed against low-ionic-strength buffer [1 mM ATP plus 5 mM Cleland Reagent, in 10 mM Tris, *p*H 8.3, (1)], and another was dialyzed against high-ionic-strength buffer [0.15 M KCl plus 5 mM Cleland Reagent in 0.1 M Tris-maleate, *p*H 6.1 (1)]. Preliminary results revealed that the patterns were rather complex, as might be expected from extracts containing polysaccharides, nucleic acids, and proteins. However, there was one peak present in the low-ionic-strength sample sedimenting at about 3*S* (at 20 C, not corrected for viscosity) which did not appear in the high-ionic-strength sample. The high-ionic-strength sample, on the other hand, showed a fast-migrating peak at about 30*S* (20 C, not corrected) which could not be observed in the low-ionic-strength sample. When the low-ionic-strength sample was recovered after the analytical run and dialyzed against high-ionic-strength buffer for 3 hr, the 3*S* peak had almost disappeared, and a 30*S* peak was found instead. The assumption that the ma-

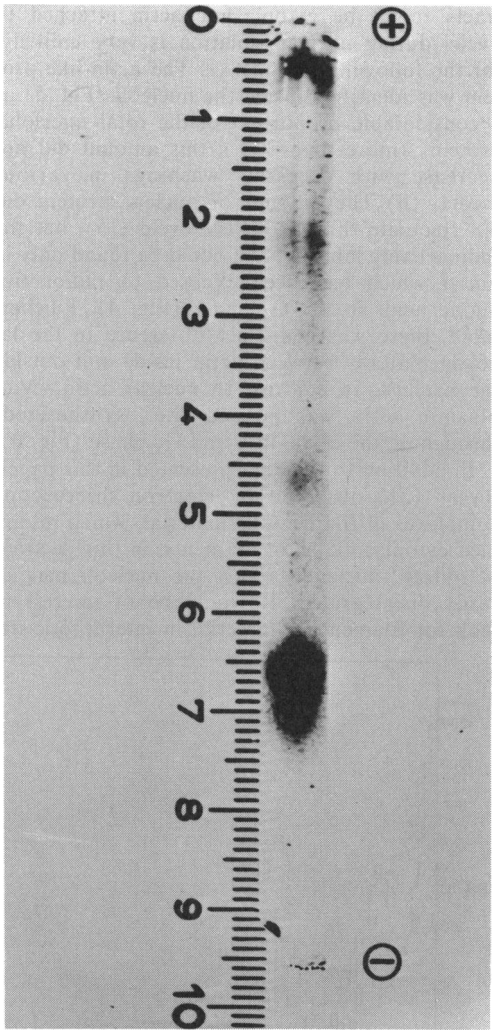


FIG. 7. *Electrophoresis in a split gel of purified actin from the cytoplasm (left side) and nuclear protein (right side). Nuclear protein used in this experiment was obtained by electrophoresis of an aqueous nuclear extract in a glycine-buffered gel and recovery of the front-running proteins. Both samples (cytoplasmic actin and the front-running nuclear protein) were dialyzed against 8.8% formic acid, plus 8 M urea and 5 mM Cleland Reagent, and layered on a split gel containing 7.5% polyacrylamide in 8.8% formic acid plus 8 M urea. Electrophoresis was carried out toward the cathode, at 30 v, for 66 hr. The electrolyte was 8.8% formic acid, plus 8 M urea, plus 0.5% 2-aminoethanethiol hydrochloride. Proteins were fixed in 10% trichloroacetic acid, stained with Coomassie Brilliant Blue (0.05%), and destained in 10% trichloroacetic acid (25). During this procedure, the gel, which was originally 80 by 6 mm, increased in size. Both protein samples show the same pattern. The majority of the material migrated in a broad peak (at the position 6.5 to 7 cm), but three minor bands are visible in the upper part of the gel.*

terial in these peaks was in fact the actin-like protein is based on the following. (i) These peaks were sensitive to a rise in temperature to 70 C or treatments with Pronase or subtilisin. Thus, they indeed represented protein. All other peaks visible in the schlieren pattern remained unchanged. (ii) No other protein was present in these extracts, as shown by SDS-urea gel electrophoresis. (iii) The peaks at 3 or 30*S*, when compared with sedimentation patterns of other proteins under the same conditions, showed the area expected at this protein concentration (1.5 mg/ml). Thus, these peaks contained most, if not all, of the protein which was present in this sample, and only very little protein could be hidden in the peaks formed by other materials present in the crude extracts.

To eliminate nucleic acids and polysaccharides, we purified the crude nuclear extracts, either by preparing the front-running proteins from the glycine-buffered gels or by precipitating acidic proteins with vincaleucoblastine, before analytical ultracentrifugation. The 3*S* peak remained unchanged by these procedures, but all attempts to cause aggregation in high-ionic-strength buffer failed. Possibly the protein denatured during these procedures, or a factor necessary for aggregation was lost.

**Synthesis of cytoplasmic actin during the mitotic cycle.** It was reported previously (1) that actin is present in large amounts (2 to 4% of all soluble proteins) in the cytoplasm of *Physarum*. Therefore, actin should form a considerable proportion of the very acidic and/or small proteins of the cytoplasm. We separated soluble cytoplasmic proteins of *Physarum* on 5% glycine-buffered gels and recovered all front-running proteins. This extract was rerun on SDS-urea gels and compared with a nuclear extract treated in the same way (Fig. 8). The most prominent peak in the stained pattern of the front-running proteins from the cytoplasm coincided with the actin-like nuclear protein. Actin could thus be identified among a special group of cytoplasmic proteins without further purification. A similar result was obtained when vincaleucoblastine precipitates of soluble cytoplasmic proteins and nuclear extracts were compared on SDS-urea gels.

The time of synthesis of actin in the cytoplasm was determined by labeling plasmodia in S and $G_2$ phase, incubating the soluble proteins with vincaleucoblastine, and separating the precipitated proteins on SDS-urea gels (Fig. 9). In many experiments with vincaleucoblastine-precipitated proteins, actin (Fig. 9, arrow) migrated as a double peak, and much material was found on top of the gels. A possible reason for this

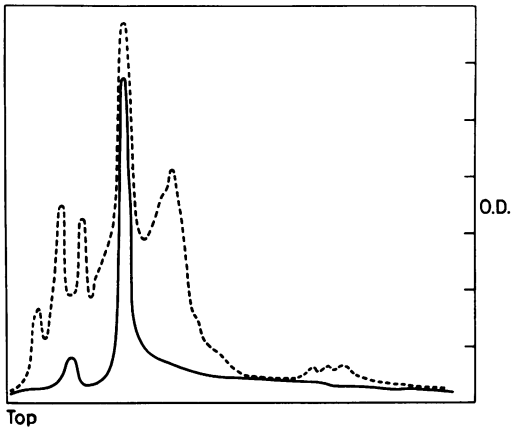


FIG. 8. *Densitometer tracings of stained patterns of cytoplasmic (dashed line) and nuclear (solid line) proteins which migrated with the front in a glycine-buffered gel, rerun in SDS-urea gels after they had been recovered from the glycine-buffered gel. Cytoplasmic and nuclear proteins in phosphate-magnesium buffer were subjected to electrophoresis in 5% polyacrylamide gels. The electrolyte was Tris-glycine buffer, containing 0.5% 2-aminoethanethiol hydrochloride. Proteins migrating with the front in this system were recovered, precipitated with acetone, redissolved in SDS-urea buffer, and separated on SDS-urea-containing 7.5% polyacrylamide gels, as described in Fig. 2.*

might be that part of the alkaloid remained irreversibly attached to the protein, thus changing its electrophoretic properties, although proteins became completely soluble during dialysis against aqueous buffer before treatment with SDS-urea. Although the total protein synthesis in *Physarum*, as measured by incorporation of radioactive amino acids into acid precipitable material, shows an approximately constant rate throughout the whole cycle, it can be seen from the radioactive patterns (Fig. 9) that cytoplasmic actin is synthesized in both the S and $G_2$ phase, but at a higher rate in $G_2$ phase.

## DISCUSSION

The data presented in this paper provide evidence for the presence of actin in the nuclei of *P. polycephalum*. The purified actin-like protein was very acidic, could be precipitated by vincaleucoblastine, and revealed a molecular weight of 44,000 when denatured with SDS and urea. Preliminary experiments gave evidence that it was able to aggregate to complexes with a sedimentation coefficient of about 30*S* at high-ionic strength. In all these properties, there was no difference when compared with slime mold actin of the cytoplasm that had been purified by coprecipitation with slime mold myosin (14). The possibility that the actin-like protein in nuclear extracts might be cytoplasmic actin attached to nuclei during nuclear isolation is very unlikely, for the following reasons. (i) The actin-like protein was identified inside the nucleoli (Fig. 5) as a considerable proportion of the total nucleolar proteins (more than 20%); this amount did not decrease with excessive washings in various media. (ii) The amount of nuclear protein did not fluctuate throughout the cycle (15), but the radioactively labeled actin could be found only in nuclei which had been exposed to radioactive amino acids in the $G_2$ phase (Fig. 4). Furthermore, there was a distinct difference in the labeling patterns between actin inside and outside the nucleus. In contrast to nuclear actin, cytoplasmic actin was labeled (i.e., synthesized) throughout the cycle, in S and $G_2$ phase (Fig. 9).

In addition to the data presented in this paper, Ryser (21) observed by electron microscopy complexes of filamentous material, which resembled cytoplasmic actin filaments, in thin sections of mitotic nuclei in which the nucleoli had already disintegrated. It would be of interest to look for filamentous material in intermitotic nu-

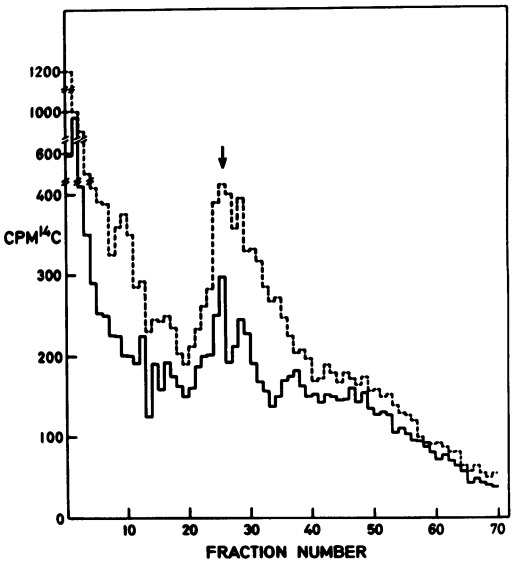


FIG. 9. *Radioactivity patterns of labeled acidic cytoplasmic proteins in SDS-urea gels. Plasmodia were labeled with $^{14}$C-amino acids in S or late $G_2$ phase, for 2.5 hr. The 100,000 × g supernatant fractions were made 5 × $10^{-3}$ M in vincaleucoblastine. Precipitated proteins were solubilized by dialysis against 67% acetic acid, precipitated again with acetone, and redissolved in SDS-urea buffer. For gels and electrophoresis conditions, see Fig. 2; for fractionation of gels and determination of radioactivity, see Fig. 4. Gels were run at 60 v, for 5.5 hr. Protein (200 μg) was layered on each gel. Solid line, proteins labeled in S phase; dashed line, proteins labeled in $G_2$ phase. Actin (arrow) was identified in these patterns by comparison with stained gels.*

clei, in which nucleoli are intact, since we have shown that actin is present in the nuclei throughout the cycle (15), and nucleoli contain all or at least a large proportion of it.

The difference in the time of labeling between nuclear and cytoplasmic actin could result from each being synthesized independently at each site, or all actin being synthesized in the cytoplasm and part of it being transported into the nuclei only during the $G_2$ phase. The second possibility could be investigated by a pulse-chase experiment with *Physarum* proteins, but thus far we have been unable to conduct a good chase experiment. The reason might be that penetration of amino acids through membranes is very slow, or that the pool sizes are very large, or a combination of these factors.

The presence of actin at two sites in the plasmodia of *Physarum* suggests that actin has two different functions in the slime mold. (i) Cytoplasmic actin, complexed with myosin, is responsible for streaming protoplasm and for the mobility of the plasmodia (27), and (ii) some is transported into the nuclei, possibly as G-actin, and may be stored in the nucleoli either in the G- or F-form. During prophase, when nucleoli disintegrate, part of the nuclear actin appears as filaments (F-actin) or may even form structures of higher molecular organization.

The function of actin as a structural protein inside the nucleus and its possible relationship to microtubules are presently unknown. Microtubules have been described in thin sections of mitotic nuclei of *Physarum* by several authors (10, 13, 21). In mitotic spindles from a number of higher eukaryotes, especially sea urchin eggs (24), a "microtubular protein," also called "tubulin," was identified as an essential component. The microtubules of the spindle apparatus in higher eukaryotes probably consist solely of this protein. Tubulin is very similar to actin in its physicochemical properties, but has a molecular weight of 55,000 to 60,000 (24). Furthermore, "microtubular protein" differs from actin in that it binds colchicine and is frequently defined by this test. Although microtubules are observed in *Physarum* spindles, it appears that such microtubules do not consist of tubulin, as no protein, similar to actin but with higher molecular weight, was found in our extracts, and no binding activity of $^3$H-colchicine was detectable in extracts of whole mold, isolated nuclei, or nucleoli (J. Toft and B. Jockusch, *unpublished data;* 5). Furthermore, mitosis in *Physarum* is not affected by colchicine.

The fact that *Physarum* apparently does not contain the tubulin found in higher eukaryotes could indicate that, in the slime mold, nuclear actin is used to form microtubules. Even though isolated *Physarum* actin (G-actin) in vitro forms only filaments (F-actin), the possibility cannot be excluded that in situ it may form microtubules, in addition to filaments. So far, it has also not been possible to reconstruct microtubules from sea urchin tubulin (23).

If microtubules of *Physarum* are not composed of actin, they must consist of "microtubular protein" which differs in properties from the tubulin found in higher eukaryotes, and nuclear actin may serve some other function. Thus, the microtubules might orient the chromosomes, while the actin filaments provide the force for moving the chromosomes, in a two-component system as proposed by Forer (8). It is also possible that actin in *Physarum* may not be directly involved with chromosomal movement during mitosis but may be concerned with the division of the nuclear membranes during telophase. Thus, the presence of actin in the nuclei may be connected with the intranuclear mitosis in the plasmodia of *Physarum*.

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