

Expression of a 66-kD Heat Shock Protein Associated with the Process of Cyst Formation of a True Slime Mold, *Physarum polycephalum*.

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ABSTRACT. Under unfavorable conditions for growth, haploid myxamoebae of *Physarum polycephalum* retracted their pseudopodia and changed their cell shape into disk-like form, after which they constructed the cell walls to form microcysts. These morphological changes of haploid cells were associated with changes in intracellular distribution of actin filaments. Staining with phalloidin showed that actin filaments were almost uniformly distributed throughout the cytoplasm of the myxamoebae. When these cells were transferred to a cyst-inducing medium, the actin structures changed into short rods or dots, after which the rods/dots disappeared in the microcysts. An incubation of the myxamoebae in the cyst-inducing medium caused the synthesis of several proteins, among which a 66-kD protein was most prominently induced. The morphological changes and the induction of the 66-kD protein was pronounced at elevated temperatures, e.g. 40°C. The 66-kD protein was not induced, however, when plasmodia of the same species were incubated at 40°C. We found that the 66-kD protein was co-precipitated with polymerized actin and bound to ATP-agarose. A double staining of the disk-shaped cells with anti-66-kD protein antibody and phalloidin revealed superimposable localization of the 66-kD protein and actin filaments in the short rods or dots. Although the induction of the 66-kD protein was enhanced at high temperatures, the protein was immunologically unrelated to the common heat shock proteins, HSP70 and HSP90, those are highly conserved during evolution. These results indicate that the 66-kD protein is a novel heat shock protein which is specifically expressed during cyst formation.

Inasmuch as environmental conditions are not constant, living organisms are frequently exposed to altered conditions including those disadvantageous for their survival. During evolution, they acquired the capability of recognizing such conditions and adapting themselves to various forms of stress. Myxamoebae of a true slime mold, *Physarum polycephalum*, change their shape from the myxamoebae to their dormant form, termed microcysts, under conditions unfavorable for their growth. Microcysts are covered with rigid cell walls composed of a galactosaminoglycan, and their intracellular physiological activities are suppressed until conditions improve (4, 6, 22). As conditions improve, dormant metabolic pathways are gradually re-activated and *Physarum* cells eventually shed their cell walls and change their shape to amoeboid form (4, 22).

In this paper, we report that the change of distribution and arrangement of actin filaments occurred concurrently with stress-induced changes in the shape of myxamoebae of *Physarum polycephalum* and that the

synthesis of a 66-kD protein was induced upon exposure of myxamoebae to stress. On the basis of the observations that the 66-kD protein is co-distributed with actin filaments and is co-precipitated with polymerized actin, we suggest the possibility that the 66-kD protein is participated in the change of actin distribution associated with the cyst formation.

MATERIALS AND METHODS

Organisms. Myxamoebae of *Physarum polycephalum*, strain J, were grown on a lawn of bacteria, *Aerobacter aerogenes*, in a nutrient agar medium (16). Ordinarily they were grown in the dark at 24°C. For heat-treatment they were incubated at 40°C.

Chemicals. [³⁵S]methionine (40.8 TBq/mmol) was obtained from ICN Biochemical Inc., Irvine, CA. ATP-agarose (1–3 μ moles of 5'-ATP/ml gel) was from Sigma Chemical Co., St. Louis, MO, horse radish peroxidase (HRP)-conjugated anti-rabbit IgG antibody and FITC-conjugated anti-rabbit IgG antibody were from Cappel, West Chester, PA. Rhodamine-phalloidin was from Molecular Probe Inc., Eugene,

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OR, CM-Sephadex, CM-Sepharose (Fast Flow), and DEAE-Sepharose (Fast Flow) were from Pharmacia, Uppsala, and Immobilon (polyvinyl membrane) from Millipore Co., Bedford, MA. Actin was prepared from acetone powder of rabbit skeletal muscle by the method of Spudich and Watt (23) and further purified by gel filtration on a Sephadex G-100 column. Congo Red was obtained from Wako Pure Chemical Industries, Tokyo. Coomassie Brilliant Blue R-250 was from Nacalai Tesque, Inc., Kyoto; Ampholine (pH 3.5–10) was from Pharmacia LKB, Bromma, Sweden; anti-HeLa HSP70 antibody was a generous gift from Dr. K. Ohtsuka, Aichi Cancer Center, Nagoya; and anti-yeast HSP90 antibody was from Dr. Y. Kimura, The Tokyo Metropolitan Institute of Medical Science.

Cyst formation study. To observe the morphological change from myxamoebae to microcysts, myxamoebae (3.3×10^7 cells/ml) were incubated in 0.025 M potassium phosphate buffer (pH 7.0) at 24°C (control) or 40°C (heat-shock condition) with shaking. At appropriate times, aliquots of cell suspension were taken out and the numbers of myxamoebae, disk-shaped cells and microcysts were counted under a microscope. Microcysts were easily distinguished from disk-shaped cells by their dimensions (see Results) and by staining with 1% Congo Red (5). Cells were also incubated in a high salt buffer (0.25 M NaCl, 2 mM MgCl₂, 0.2 mM CaCl₂, 7 mM KH₂PO₄, pH 7.0) or in a buffer with high osmolarity (0.5 M mannose in 0.025 M potassium phosphate buffer, pH 7.0), and observation of morphological changes was performed with a microscope.

Labeling of newly synthesized proteins. Myxamoebae grown at 24°C for 5 days were washed several times with 0.025 M potassium phosphate buffer (pH 7.0) to remove bacteria, then collected by centrifugation. The number of cells was adjusted to 3.3×10^7 cells/ml by the addition of the above buffer. The cell suspension was incubated at 24°C (control) or 40°C with shaking for appropriate time. Alternatively, the cells were incubated in the high salt buffer or in the hypertonic buffer as described above. Cells were incubated with 1.1 MBq/ml of [³⁵S]methionine in the incubation buffer for appropriate time. At each time, an aliquot of the cell culture was pipetted and washed four times with 5 vol. of the buffer containing 1 M unlabeled methionine. The washed cells were then lysed in sodium dodecyl sulfate (SDS) sample buffer (11) for SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Labeling and analysis of *Aerobacter* proteins were also performed as described above.

Gel electrophoresis and fluorography. One dimensional SDS-PAGE was performed according to the method of Laemmli (11) using 10% polyacrylamide gels, or 5–20% polyacrylamide gradient gels. To visualize protein bands, gels were stained with Coomassie Brilliant Blue (CBB). For fluorography, the slab gel was fixed with 40% methanol–10% acetic acid for 1 h, washed with distilled water for 30 min, and soaked in 1 M sodium salicylate for 30 min. The slab gel was then dried on Whatman 3MM filter paper under vacuo. Fluor-

ography was performed with Kodak X-Omat film at –80°C. Two dimensional polyacrylamide gel electrophoresis (2D-PAGE) was performed according to O'Farrell (20).

Preparation of the anti-66kD protein antibody. Heat-treated myxamoebae (30-min treatment) were collected by centrifugation, suspended in 5 vol. of homogenizing buffer (10 mM Tris-HCl, pH 8.0, 5 mM 2-mercaptoethanol, 5 mM sodium pyrophosphate, 0.5 mM PMSF, 1 mM EDTA and 0.2 mM KCl), and sonicated for 5 min by a Branson Sonifier. The homogenate was centrifuged at $45,000 \times g$ for 30 min and the supernatant was applied to a CM-Sephadex column equilibrated with the homogenizing buffer. The column was washed with the same buffer and adsorbed proteins were eluted with a linear gradient of 0–1.0 M NaCl in the homogenizing buffer. After the analysis by SDS-PAGE, fractions containing the 66-kD protein which was bound to CM-Sephadex were pooled and chromatographed on an ATP-agarose column equilibrated with TEM buffer (20 mM Tris-acetate, pH 7.5, 0.1 mM EDTA, 15 mM 2-mercaptoethanol). After washing with TEM buffer, adsorbed proteins were eluted with 3 mM ATP in the same buffer.

The fraction containing the 66-kD protein which was weakly bound to ATP agarose was electrophoresed in SDS-PAGE. The 66-kD protein band was excised from the gels with a razor blade, after which the gel pieces were washed with phosphate-buffered saline (PBS), homogenized in a small volume of

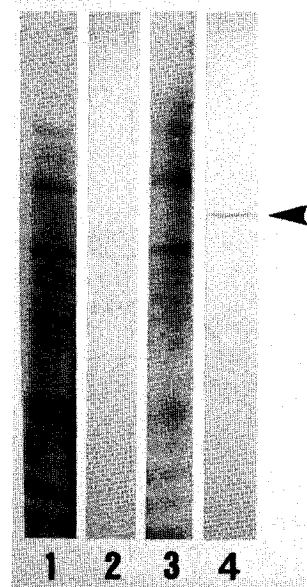


Fig. 1. Specificity of affinity-purified anti-66 kD protein antibody. Cells were incubated at 24°C (lanes 1 and 2) or at 40°C (lanes 3 and 4) for 30 min, and the total cell extracts were electrophoresed using 10% polyacrylamide gel and proteins were transferred to Immobilon membrane. They were then stained with CBB (lanes 1 and 3) or anti-66 kD protein antibody (lanes 2 and 4). On each lane, the lysate containing approximately 50 µg protein was applied. Arrowhead shows a position of 66-kD protein.

PBS, and emulsified with an equal volume of Freund's complete adjuvant. A rabbit was immunized with about 0.1 mg each of the 66-kD protein every three weeks. After the fourth immunization, the rabbit was bled. Affinity purification of the antibody was carried out using Immobilon sheet which bound the 66-kD protein, and stored at -80°C . The affinity-purified antibody was specific for the heat-inducible 66-kD protein as shown in Fig. 1.

Immunoblotting. Immunoblotting was performed by the method of Towbin *et al.* (24). Membranes were incubated either with anti-66 kD antibody, anti-HSP70 antibody or anti-HSP90 antibody as the first antibody. HRP-conjugated anti-rabbit IgG antibody was used as the second antibody. HRP reaction was performed with 1–4-chloronaphtol and H_2O_2 .

Immunofluorescence staining. Myxoamoebae grown on bacteria lawn for 5 days were harvested and washed as described. Washed myxoamoebae were suspended in the same buffer and placed onto coverslips coated with 1 mg/ml of poly-L-lysine. Heat treatment of the cells on coverslips was done at 40°C for 30 min or 60 min in a dark moist box. Control cells on coverslips were kept at 24°C in the dark moist box. After the treatment, cells were fixed by immersing coverslips in 3.7% formaldehyde/PBS (pH 7.0) at room temperature for 10 min. After washing twice with PBS, the coverslips were immersed in 0.5% Triton X-100 for 5 min and washed with PBS again.

To stain actin filaments, Triton-permeabilized cells were incubated with rhodamine-phalloidin at 37°C for 30 min. Immunofluorescence staining of the 66-kD protein was performed to determine the localization of this protein in the cell. Cells attached to coverslips were incubated with the affinity-purified anti-66 kD protein antibody at 37°C for 30 min. After washing with PBS, cells were incubated with FITC-conjugated anti-rabbit IgG antibody at 37°C for 30 min. Immunofluorescence was observed by a Nikon Optiphot fluorescence microscope.

Co-precipitation with actin. Samples prepared from myxoamoebae were dialyzed against homogenizing buffer and centrifuged at $100,000 \times g$ for 1 h to remove insoluble materials. The supernatants were added to an incubation mixture consisting of 30 mM Hepes (pH 7.0), 10 mM KCl, 1 mM MgCl_2 , 0.04 mM ATP, and 0.12 mg/ml rabbit muscle actin. The mixtures were incubated at 0°C for 1 h, and centrifuged at $100,000 \times g$ at 2°C for 2 h, after which the supernatants and the precipitates were analyzed by SDS-PAGE.

RESULTS

Cyst formation under stressful conditions. Myxoamoebae of *P. polycephalum* formed microcysts when they are exposed to different forms of stress such as heat shock, starvation, high osmotic pressure, or high salt treatment. Heat treatment at 40°C was most effective for the induction of the cyst formation. The time course of cyst formation at 40°C is shown in Fig. 2. Most of

the cells were amoeboid during incubation at 24°C for 90 min. Upon shift up of the temperature to 40°C , disk-shaped cells and microcysts appeared, and almost no amoeboid cells remained 15 min later. A population of disk-shaped cells initially increased with time. Disk-shaped cells with flattened morphology had no cell walls, and were subsequently transformed to microcysts. Disk-shaped cells and microcysts were easily distinguished by their characteristics as described in Materials and Methods. The microcyst formation was also induced by starvation, high osmotic pressure or high salt conditions. The cyst formation under these conditions occurred much slower than that induced by heat treatment (data not shown).

Changes in actin distribution during microcyst formation. We examined the distribution of actin filaments in cells in the course of cyst formation induced by heat treatment. Intracellular actin filaments were visualized by fluorescence microscopy using rhodamine-conjugated phalloidin (Fig. 3). In myxoamoebae, actin was diffusely distributed throughout the cells, and, in addition, concentrated in microspikes of pseudopodia (Fig. 3a, d). In disk-shaped cells with a diameter of about $8 \mu\text{m}$, numerous short, rod-like structures or dots of F-actin were observed at the peripheral region of the cells (Fig. 3b, e). In microcyst cells with a diameter of about

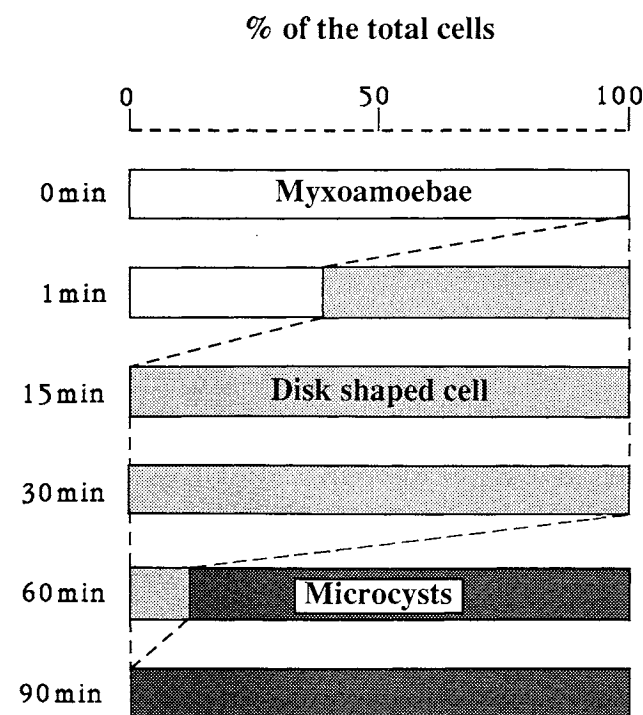


Fig. 2. Cyst formation caused by heat treatment at 40°C . Numbers of amoeboid cells, disk-shaped cells and microcysts were counted under the microscope. More than 1,000 cells were counted each time.

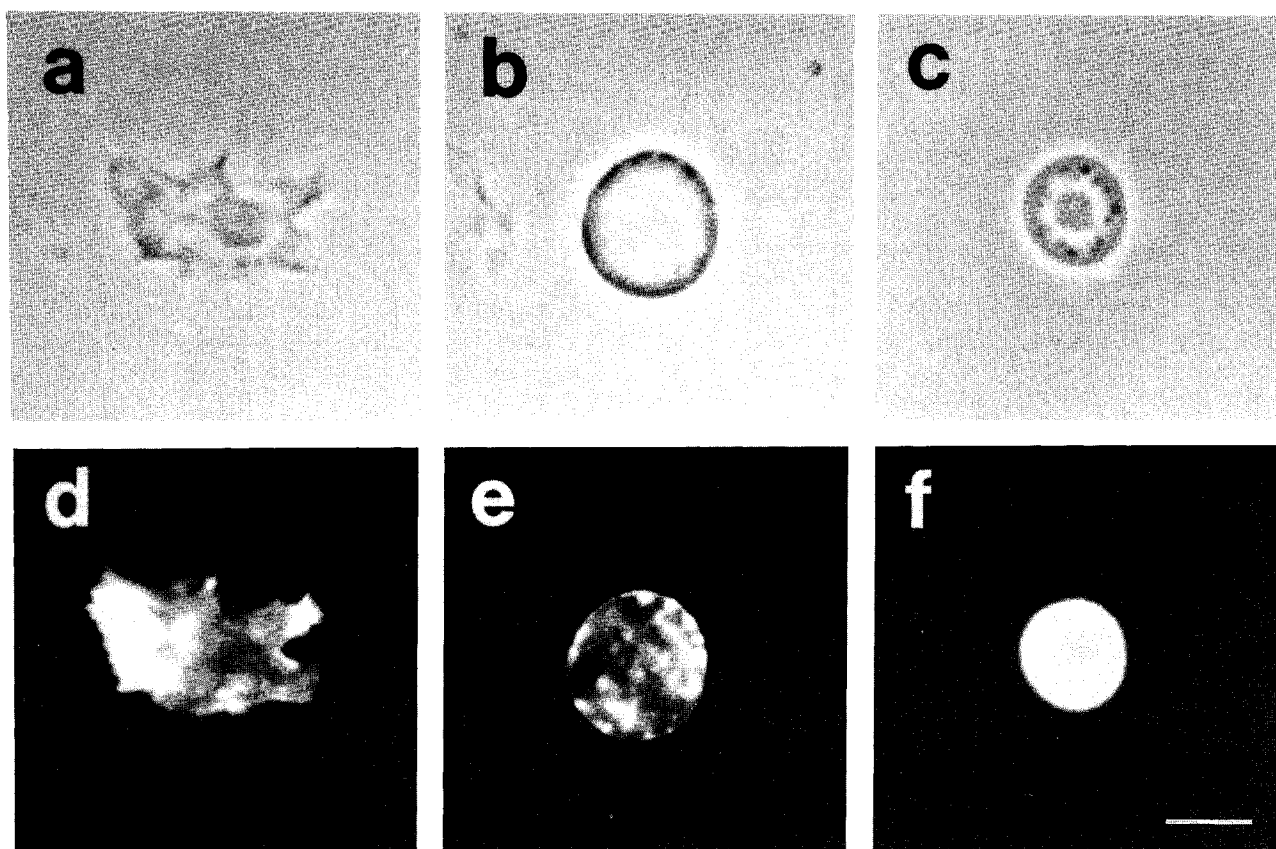


Fig. 3. Localization of actin filaments in the three types of cells. Actin filaments were stained with rhodamine-phalloidin. a, d, an amoeboid cell; b, e, a disk-shaped cell; c, f, microcyst. Cells were incubated at 24°C (a, d) or at 40°C (b, c, e, f). The upper row (a-c), phase contrast; the lower row (d-f), fluorescence; bar, 5 μ m.

5–6 μ m, which have cell walls, actin rods/dots disappeared and microfilaments were localized throughout the whole cells (Fig. 3c, f). Similar changes in distribution of actin filaments were also induced under other stress conditions such as starvation, high osmotic pressure or high salt treatment. The changes of distribution of actin filaments were always associated with the process of cyst formation.

Synthesis of stress proteins. Since cells were incubated at 40°C during cyst formation, stress proteins could be synthesized in these cells. Myxamoebae were incubated at 24°C or 40°C for various periods in 0.025 M potassium phosphate buffer containing [35 S]methionine. The cells were lysed and subjected to SDS-PAGE followed by fluorography (Fig. 4a and c). When incubated at 24°C, a variety of proteins including actin were synthesized (Fig. 4a). At 40°C, the synthesis of the 66-kD protein became prominent soon after the temperature shift-up (Fig. 4c) and the synthesis of this protein continued while the disk-shaped cells were present. The synthesis of 90-kD and 76-kD proteins also increased significantly at 40°C. The syntheses of other proteins

were decreased to some extent at 40°C.

The expression of the 66-kD protein was also examined with immunostaining. The prepared antibody was specific for the heat-inducible 66-kD protein (Fig. 1). Even when the cell lysate was analyzed by 2D-PAGE, only one spot was stained with this antibody. Fig. 4b and Fig. 4d clearly show that the 66-kD protein was expressed rapidly at 40°C but not at 24°C. The synthesis of the 66-kD protein was always associated with the appearance of disk-shaped cells.

Aerobacter aerogenes cells were incubated at 24°C and 40°C for various periods in the presence of [35 S]methionine, to examine the possibility that the 66-kD protein might be derived from the feed. The lysates of *A. aerogenes* cells were electrophoresed and blotted to Immobilon. The membrane was stained with anti-66 kD protein antibody and also analyzed by autoradiography. The results clearly showed that the 66-kD protein was not synthesized at all in *A. aerogenes* cells at 40°C (data not shown).

When the diploid plasmodia were exposed to heat shock, no synthesis of 66-kD protein was detected by

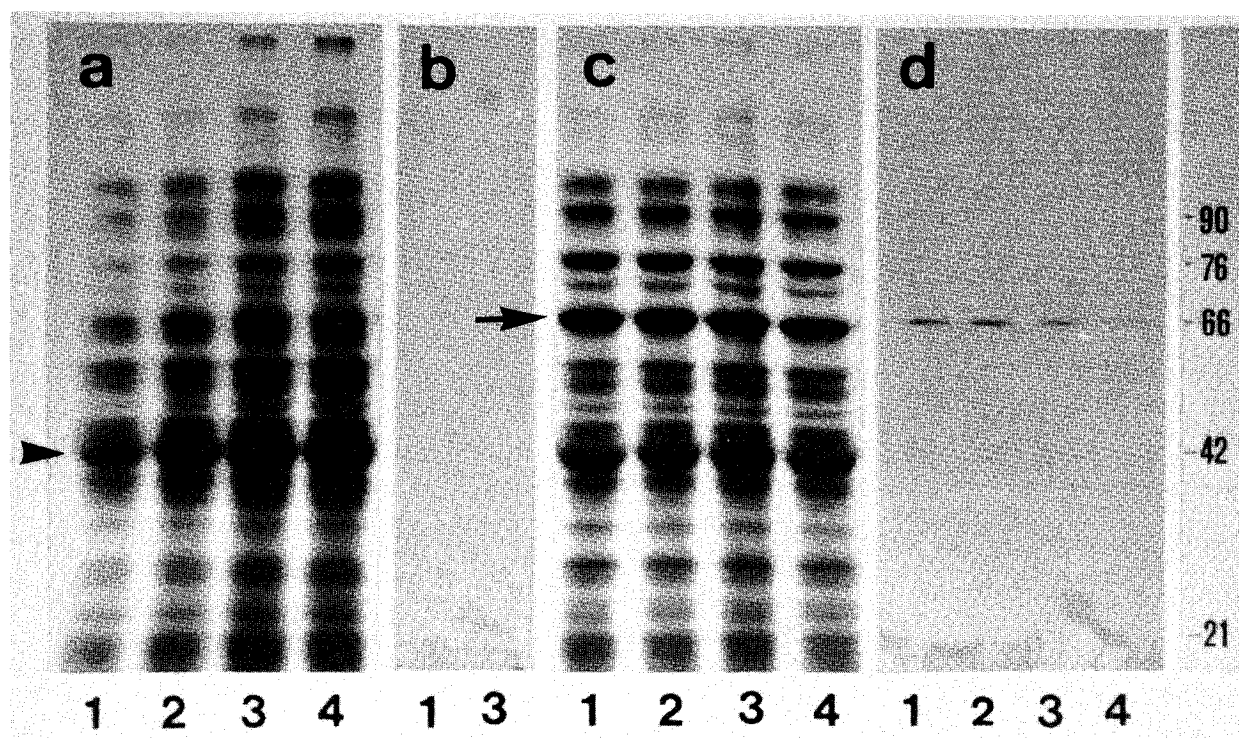


Fig. 4. Protein synthesis and expression of the 66-kD protein in myxamoebae of *Physarum polycephalum* under heat-shocked condition. Cells were incubated at 24°C (a) or 40°C (c) in the presence of [³⁵S]methionine, and the total cell extracts were electrophoresed with 10% polyacrylamide gel and fluorograms of polyacrylamide gels were made. On each lane, 100,000 cpm were applied. Cells incubated at 24°C (b) or 40°C (d) without radioactive substance were also lysed and the total extracts were electrophoresed, transferred to Immobilon membrane and stained with the anti-66 kD protein antibody. Incubation times are 15 min (lane 1), 30 min (lane 2), 60 min (lane 3) and 90 min (lane 4), respectively. Arrowhead, actin; arrow, 66-kD protein.

either an experiment of [³⁵S]methionine incorporation or an immunostaining with anti-66 kD protein antibody (Fig. 5). As the plasmodia could not survive at 40°C, this experiment was done at 35°C.

Co-precipitation experiment with actin. To search for protein(s) which might be involved in the change of distribution of actin during cyst formation, proteins co-precipitated with actin filaments were investigated (Fig. 6). Heat-shocked cells were labeled with [³⁵S]methionine, and the cell extracts were mixed with rabbit skeletal muscle actin and stood at 0°C for 1 h under the actin polymerization condition. Polymerized actin and its associated proteins were then pelleted by centrifugation. Four proteins with molecular mass of 90-kD, 66-kD, 25-kD and 21-kD were precipitated together with polymerized actin mainly, suggesting that these proteins had a strong actin-binding activity. Among these proteins, 90-kD and 66-kD proteins were induced by heat treatment. The co-precipitation results were not affected by EDTA or ATP. The 66-kD protein precipitated with actin filaments reacted with the anti-66-kD protein antibody.

ATP-binding proteins. As the proteins belong to HSP70-family are known to have an ATP-binding activity and a weak ATPase activity (12), we examined the

ATP-binding activity of heat-induced 66-kD protein of myxamoebae of *P. polycephalum*. Partially purified samples after CM-Sephadex column chromatography were applied on an ATP-agarose column. The 66-kD protein bound to the ATP-agarose, and was eluted with 3 mM ATP together with some other proteins (Fig. 7, lane 1). Proteins eluted from the ATP-agarose column were then subjected to the co-precipitation with polymerized actin. The 66-kD protein was co-precipitated (Fig. 7, lane 3). Immunoblot analysis revealed that the ATP-binding 66-kD protein co-precipitated with polymerized actin reacted with the anti-66-kD protein antibody. Taken together, these results strongly suggest that the 66-kD protein has the ability to bind to both actin and ATP.

Intracellular distribution of the 66-kD protein. The distribution of the 66-kD protein in the heat-shocked cells was examined by indirect immunofluorescence staining. A double immunofluorescence staining experiment was performed using disk-shaped cells with the anti-66 kD protein antibody and rhodamine-phalloidin (Fig. 8). The results showed that the 66-kD protein is distributed as short rods or dots which were also stained with rhodamine-phalloidin (Fig. 8). In myxamoebae

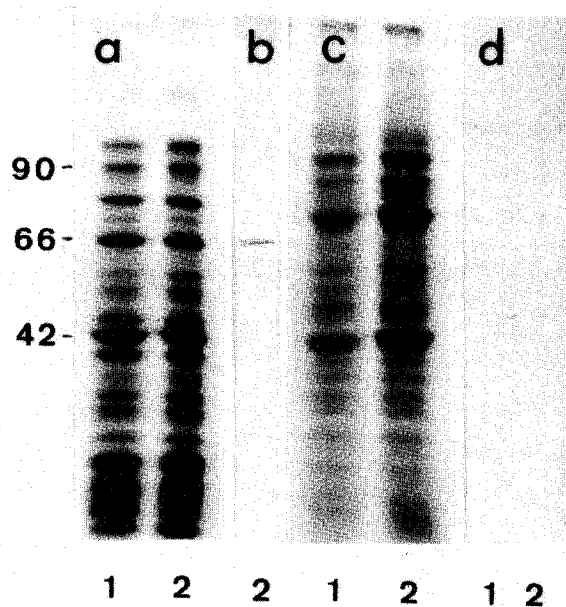


Fig. 5. Fluorography and immunoblotting of newly synthesized proteins in plasmodia under heat-shocked condition. Plasmodia (c) or myxoamoebae (a) were incubated at 35°C in the presence of [³⁵S]methionine, and the total cell extracts were electrophoresed and fluorography was performed as described. For labeling of plasmodia, cells were incubated with 3.7 MBq/ml of radioactive substance instead of 1.1 MBq/ml. On each lane, about 100,000 cpm were applied. Without radioactive methionine, plasmodia (d) or myxoamoebae (b) were also incubated at 35°C and the total extracts were electrophoresed and immunoblotting with anti-66 kD protein antibody was carried out. Incubation periods are 30 min (lane 1) and 60 min (lane 2), respectively.

and microcysts, no structures were significantly stained with the antibody (data not shown).

Comparison of *Physarum* 66-kD protein with other HSPs in their cross-reactivity with some antibodies. To determine the immunological properties of the 66-kD protein, the cross-reactivity of the protein with the anti-HeLa HSP70 and anti-yeast HSP90 antibodies was examined (Fig. 9). After SDS-PAGE, proteins were blotted to Immobilon. Each lane was cut longitudinally into two parts; the left part was stained with anti-66 kD protein antibody and the right part was stained with either anti-HSP70 or anti-HSP90 antibody. The 66-kD protein did not cross-react with anti-HSP70 or anti-HSP90.

DISCUSSION

In myxoamoebae of *P. polycephalum*, drastic changes in the organization of actin filaments was induced under stressful conditions. Actin filaments formed short, rod-like structures or dots, which were distributed in the cytoplasm, particularly in the cell periphery.

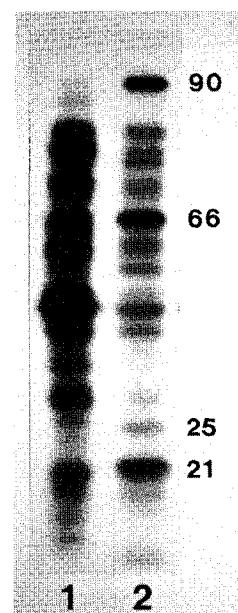


Fig. 6. Co-precipitation of cell extracts with polymerized actin. The extract derived from the cells which were labeled with [³⁵S]methionine at 40°C for 30 min was mixed with the reaction mixture containing actin and incubated. The mixture was then centrifuged at 100,000 × g for 2 h. The supernatant (lane 1) and the pellet (lane 2) were analyzed by SDS-PAGE, and fluorogram was made.

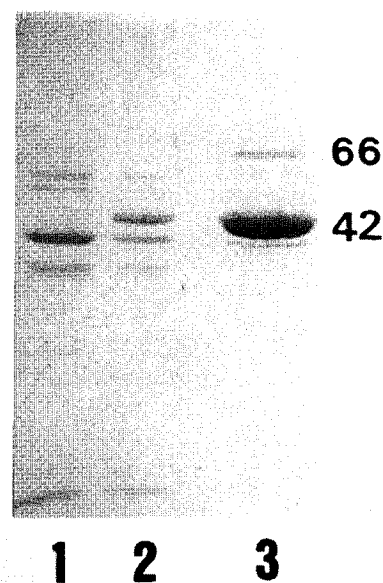


Fig. 7. Binding of 66-kD protein to ATP-agarose. The sample, which was partially purified by CM-Sephadex column chromatography, was applied to ATP-agarose. Adsorbed proteins were eluted with 3 mM ATP and analyzed by SDS-PAGE (lane 1). The ATP-binding proteins were then incubated with actin to examine the actin-binding activity. The polyacrylamide gel was stained with CBB. Lane 1, ATP-agarose binding proteins; lane 2, supernatants from the co-precipitation experiment; lane 3, pellets from the co-precipitation experiment.

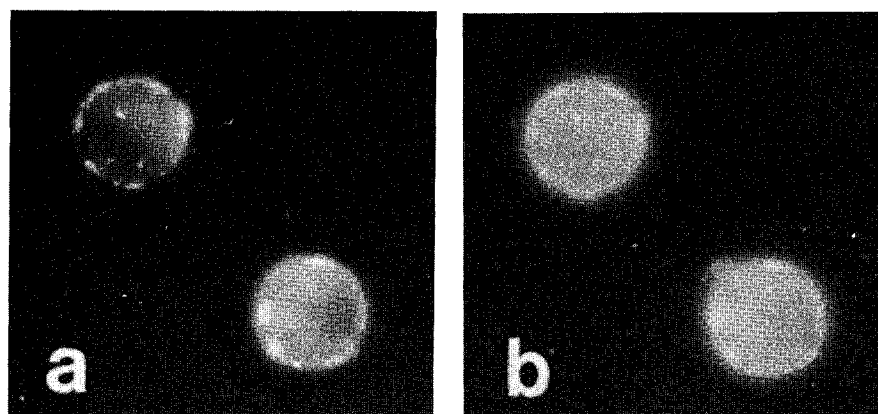


Fig. 8. Co-localization of actin and the 66-kD protein revealed by double-labeled immunofluorescence. The distribution of actin filaments in the disk-shaped cells induced by heat stress at 40°C for 30 min were visualized with rhodamine-phalloidin (a) and the anti-66 kD protein antibody (b).

The structures of actin observed in heat-treated *Physarum* cells resemble cortical actin patches of the budding yeast, *Saccharomyces cerevisiae* (1, 9, 13). In the yeast, cortical actin patches are distributed under membrane growth sites, particularly in buds, where newly

synthesized membrane vesicles are inserted into the cell surface membrane. As the membrane synthesis does not occur during the cyst formation, the function of actin rods/dots in heat-treated *Physarum* cells may differ from the cortical actin patches.

Iida *et al.* (7, 8) have reported the formation of intranuclear actin rods induced by heat treatment in cultured mammalian cells. No cytoplasmic actin rods were induced in these cells upon heat shock, however. Conversely, cytoplasmic actin rods were induced when *Physarum* cells were incubated at 40°C as shown in this report. Cytoplasmic actin rods were induced in cultured mammalian cells when they were incubated in a low K⁺-ion buffer, or treated with a low concentration of non-ionic detergents (8). All of the actin rod structures induced in cultured mammalian cells have been shown to contain cofilin, an actin-modulating protein (17), as a component (19). Cofilin regulates the polymerization state of actin in a pH-dependent manner (28). We have not investigated whether the rod/dot-structures observed in disk-like *Physarum* cells contain cofilin as a component, but the rod/dot-actin structures may differ from the actin/cofilin rods induced mammalian cells because the former structures were stained with phalloidin but the latter was not (7, 8). The relationship of the actin rod/dot-structures described in this paper to the actin rods previously reported remains to be clarified.

Under stress conditions, myxamoebae were stimulated to synthesize three proteins of 66-kD, 76-kD and 90-kD, among which the 66-kD protein was the most prominent. In the plasmodia of *Physarum polycephalum*, the induction of four proteins, 105-kD, 82-kD, 74-kD and 69-kD by heat shock, has been reported by Wright and Tollon (26), though these proteins have not been well characterized. In the heat-shocked plasmodia, no synthesis of the 66-kD protein occurred, and the in-

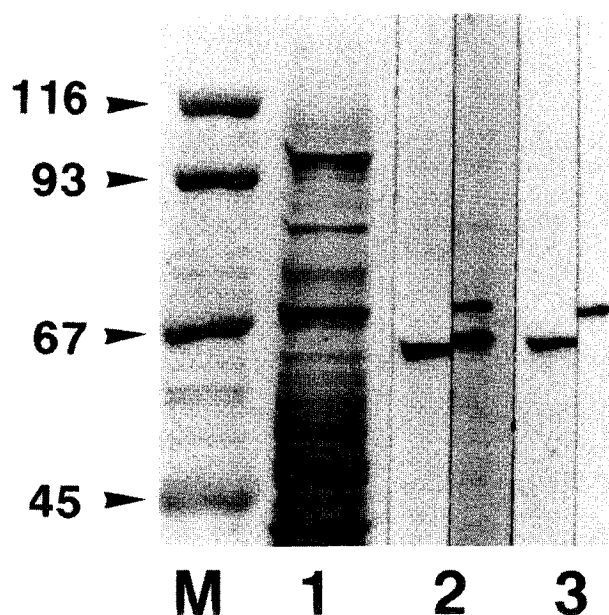


Fig. 9. The cross-reactivity of 66-kD protein with the antibodies directed against HSP70 and HSP90. The cell extracts of myxamoebae incubated at 40°C were electrophoresed in 5–20% polyacrylamide gradient gel and proteins were transferred to an Immobilon membrane. M, marker proteins; lanes 1–3, total extract from *Physarum* myxamoebae. Lane 1, stained with CBB; lanes 2 and 3, immunostained with specific antibodies. Lane 2, the left half, anti-66 kD protein antibody, the right half, anti-HSP70 antibody. Lane 3, the left half, anti-66 kD protein antibody, the right half, anti-HSP90 antibody.

duction of actin rods was not observed. On the basis of these findings, we assume that the 66-kD protein might play a role in the differentiation from myxoamoebae to microcysts. We have shown that the 66-kD protein has an actin-binding activity. In addition, the results revealed that the 66-kD protein and F-actin were colocalized in the disk-like cells. The 66-kD protein may be a modulating protein of actin structures which is induced during transformation to microcysts.

The 66-kD protein resembles HSP70 in its ATP-binding activity (2, 14, 25), and is also similar to HSP90 in its actin-binding activity (10, 27). Recently, it has been shown that HSP90 also possesses an ATP-binding site (3). Despite these similarities in addition to the heat inducibility of the three proteins, the 66-kD protein is antigenically unrelated to HSP70 or HSP90.

Finally, it should be noted that the 66-kD protein is not included in the category of standard heat shock proteins. The induction by heat or by other form of stress of the 66-kD protein was observed in myxoamoebae but not in plasmodia. This suggests that the control mechanism operating in the expression of the 66-kD protein must differ from those of standard heat shock proteins.

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