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A Novel 66-kDa Stress Protein, p66, Associated with the Process of Cyst Formation of *Physarum polycephalum* Is a *Physarum* Homologue of a Yeast Actin-Interacting Protein, AIP1[1]

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When exposed to various stresses including heat shock, myxoamoebae, growing haploid cells of *Physarum polycephalum*, show marked morphological changes and consequently become disk-shaped microcysts. We have found that p66 is induced exclusively in the course of microcyst formation and has an actin-binding activity. In this study, we purified p66 to homogeneity and isolated a p66 cDNA. The deduced protein sequence contained 601 amino acids and showed 31% identity to a yeast actin-interacting protein, AIP1. Northern blot analysis revealed that the amount of p66 mRNA was significantly increased by heat shock in myxoamoebae but not in plasmodia. Thus, p66 seems to be a developmentally-expressed stress protein which regulates the rearrangement of actin organization during microcyst formation in *P. polycephalum*.

Key words: actin-binding protein, cDNA cloning, heat shock, *Physarum polycephalum*, stress response.

Cell differentiation often accompanies dynamic morphological changes which require gross rearrangement of cytoskeletons. Actin filaments constitute a major part of cytoskeletons and undergo remarkable rearrangements during the cell cycle and cell differentiation. In each case, the signaling cascade involves crucial factors acting at a late step that directly transfer information to actin. For example, haploid cells of the budding yeast *Saccharomyces cerevisiae* respond to mating pheromones with polarized growth toward the mating partner. This morphological response requires the function of Bem1p, which contains two SH3 domains and directly links the MAP kinase cascade with actin (*1*). The 15-21-kDa actin-binding protein cofilin has been shown to be essential for cell growth in the budding yeast and in *Dictyostelium discoideum* (*2-4*) and is suggested to be responsible for the rapid turnover of actin filaments *in vivo* (*5-7*). Dephosphorylation of Ser-3 of cofilin is essential for its function on actin (*8*), and several stresses, mitogens, and stimuli of differentiation are known to induce dephosphorylation of cofilin (*9-12*).

Haploid myxoamoebae of a true slime mold, *Physarum polycephalum*, show remarkable morphological changes when they encounter stresses such as increased temperature, high salt, starvation, metabolic inhibitors, or transition metals. They retract pseudopodia, assume a disk-like form, accompanied by marked changes in the actin filament organization, and construct cell walls to differentiate into their dormant form, so-called microcysts (*13, 14*). We have found that the synthesis of a 66-kDa protein, p66, was induced in myxoamoebae immediately after such stresses (*13*). The p66 was co-localized with short bundles of actin filaments (short rods or dots) occurring in the microcysts, and was co-precipitated with polymerized actin *in vitro*. Therefore, p66 may play a crucial role in the reorganization of the actin cytoskeleton associated with stress-induced microcyst formation.

Living organisms including the true slime mold produce so-called stress proteins or heat-shock proteins (HSPs) to protect themselves from unfavorable conditions (*15, 16*). Though p66 was induced by heat-shock and was a major protein product synthesized after heat-shock, it was not recognized by antibodies against the common heat shock proteins, hsp70 or hsp90. Further, p66 was not induced in diploid plasmodia of the same species exposed to the same stress conditions (*13*). These results suggested that p66 is a novel stress protein specifically expressed during microcyst formation in haploid *Physarum* cells.

In the present study, we purified p66 biochemically from heat-treated myxoamoebae, isolated p66 cDNA, and showed that p66 is a *Physarum* homologue of AIP1, an actin-interacting protein of the budding yeast, *S. cerevisiae*.

MATERIALS AND METHODS

Cell Cultures and Heat Treatment—Myxoamoebae of *P. polycephalum* were grown in the dark at 24°C on a lawn of

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Abbreviations: CBB, Coomassie Brilliant Blue R-250; IPTG, Isopropyl-β-D-thiogalactopyranoside; ORF, open reading frame; RT-PCR, reverse transcription-polymerase chain reaction.

bacteria, *Aerobacter aerogenes*, in a nutrient agar medium (*17*). For heat-shock treatment, they were harvested, washed several times with 25 mM potassium phosphate buffer, pH 7.0, to remove bacteria, and incubated in the dark at 40°C for 30 min. Plasmodia of *Physarum* were cultured in the semi-defined media according to Daniel and Rusch (*18*) in the dark at 24°C. For heat-shock treatment, they were harvested, resuspended in 25 mM potassium phosphate buffer, pH 7.0, and incubated in the dark at 40°C for 30 min.

Purification of p66—p66 was extracted and purified from heat-treated (at 40°C for 30 min) myxoamoebae. Heat-treated cells (4.8×10^9) were harvested in homogenizing buffer (10 mM K-phosphate buffer, pH 7.0, 1 mM EDTA, 5 mM 2-mercaptoethanol, 5 mM Na-pyrophosphate, and 0.1 mM KCl) and lysed by homogenization followed by sonication. The homogenate was centrifuged at $40,000 \times g$ for 30 min, and 120 ml of the supernatant (576 mg total protein) was applied on a CM-Sephadex column equilibrated with the homogenizing buffer. p66 was weakly bound to CM-Sephadex and was recovered in a wash fraction (total 29.5 mg protein). Actin was not adsorbed to this resin under these conditions. The fraction containing p66 was dialyzed against 10 mM Tris-HCl, pH 8.0, 5 mM 2-mercaptoethanol, and applied on a column of DEAE-Sephadex equilibrated with the same buffer. p66 was not adsorbed to DEAE-Sephadex and was recovered in the flow-through fraction (total 5.4 mg protein). The p66-containing fraction was dialyzed against 25 mM PIPES buffer, pH 6.6, containing 5 mM 2-mercaptoethanol, then chromatographed on a Phospho-cellulose column. p66 was recovered in the flow-through fraction (total 1.8 mg protein). This fraction was dialyzed against 20 mM K-phosphate buffer, pH 7.0, containing 5 mM 2-mercaptoethanol, then chromatographed on a hydroxylapatite column equilibrated with the same buffer. p66 was adsorbed to hydroxylapatite and eluted at 150 mM K-phosphate. Hydroxylapatite column chromatography was repeated twice, and 0.09 mg of p66 was purified as a single protein as judged by SDS-PAGE (*19*) stained with CBB (Fig. 2).

Purification of Physarum Actin—The flow-through fraction from CM-Sephadex described above was dialyzed against 10 mM K-phosphate buffer, pH 7.0, and 5 mM 2-mercaptoethanol. The dialysate was centrifuged at $30,000 \times g$ for 30 min, the precipitate was dissolved in 10 mM Tris-malate buffer, pH 7.0, containing 0.5 M KCl, and solubilized proteins were dialyzed against distilled water. The resultant precipitate was dissolved in 10 mM Tris-malate, pH 7.0, containing 5 mM 2-mercaptoethanol, 2 mM $MgCl_2$, and 4 mM ATP, then centrifuged at $100,000 \times g$ for 2 h. The pellet was dissolved in 2 mM Na-hydrocarbonate buffer, pH 8.0, containing 0.6 M KI, 5 mM ATP, 10 mM 2-mercaptoethanol, and dialyzed against the same buffer containing ATP and 2-mercaptoethanol. The dialysate was centrifuged at $100,000 \times g$ for 1 h, and *Physarum*

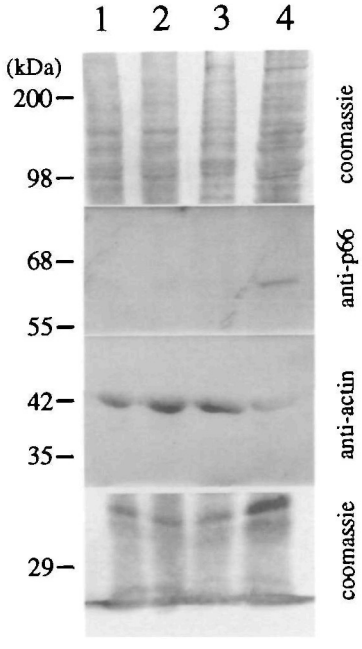

Fig. 1. **Stage-specific expression of p66.** A protein blot was divided into four sheets and each sheet was stained with either CBB, anti-p66 antibody, or anti-actin monoclonal antibody (C4) as shown. A total of 37 μg of protein was loaded on each lane. Lane 1: Crude cell extract from plasmodia (diploid) cells. Lane 2: Heat-treated (at 40°C for 30 min) plasmodia. Lane 3: Myxoamoebae (haploid) cells. Lane 4: Heat-treated (at 40°C for 30 min) haploid cells. Molecular mass standards: myosin (200 kDa), phosphorylase *b* (98 kDa), bovine serum albumin (68 kDa), tubulin (55 kDa), actin (42 kDa), glyceraldehyde-3-phosphate dehydrogenase (35 kDa), and carbonic anhydrase (29 kDa).

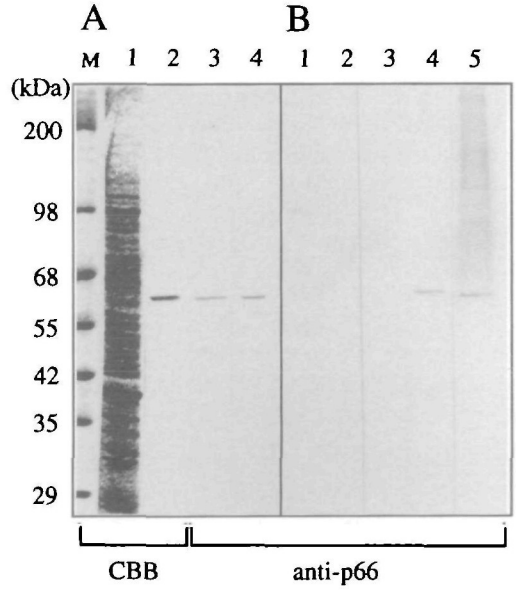

Fig. 2. **Purification of p66 from *Physarum* myxoamoebae and synthesis of recombinant p66 in *E. coli*.** (A) A crude *Physarum* cell extract and purified p66 were resolved by SDS-PAGE and Western blotted with anti-p66 antibody. Lane 1: Crude cell extract from heat-treated *Physarum* myxoamoebae stained for total protein with CBB. Lane 3: Corresponding immunoblot probed with anti-p66 antibody. Lane 2: Purified p66 stained with CBB. Lane 4: Corresponding immunoblot. (B) ORF product synthesized in *E. coli* reacted with anti-p66 antibody, showing that the isolated cDNA clone indeed encodes for p66. *E. coli*, JM109, harboring pUC19(ORF) (see "MATERIALS AND METHODS") or control plasmid in which ORF follows lacZ alpha peptide but in out-of-frame fashion grown with or without IPTG were lyzed in SDS sample buffer and analyzed by immunoblot. Lane 1: Control cells (IPTG−). Lane 2: Control cells (IPTG+). Lane 3: Cells with pUC19(ORF) (IPTG−). Lane 4: Cells with pUC19(ORF) (IPTG+). Lane 5: *Physarum* amoebae extract.

myxoamoebae actin was obtained in the supernatant. This actin fraction consisted of a single protein as judged by CBB staining (Fig. 3, lane 2).

Co-Precipitation of p66 and Actin—Binding of p66 to actin was examined by co-precipitation in a polymerization 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 *Physarum* actin. The mixture was incubated at 0°C for 1 h and centrifuged at 100,000 × *g* at 2°C for 2 h, then the supernatant and the precipitate were analyzed by SDS-PAGE.

Determination of Partial Amino Acid Sequence of p66—The purified p66 was further separated by SDS-PAGE, and the p66 band was excised and treated with 1 mg/ml lysyl-endopeptidase in 0.1 M Tris-HCl, pH 9.0, and 1% SDS, at 37°C overnight as described (*20*). Peptides were separated by successive high performance liquid chromatographies with DEAE-Toyopearl and Wakosil 5C18. The separated peptides were sequenced by use of Applied Biosystems Model 470A and Model 120A PTH amino acid analyzers.

Primers for RT-PCR and cDNA Cloning of p66—RNA from *Physarum* myxoamoebae heat-treated at 40°C for 15 min was extracted with guanidine thiocyanate followed by centrifugation in cesium chloride solution (*21*). mRNA was purified from the total RNA fraction using Oligotex™-dT30 (TaKaRa) according to the manufacturer's instructions. From the amino acid sequences of several peptides derived from p66, two sequences, DIYYEHA and KIWDDK (see Fig. 5), were used to design RT-PCR primers. Of two combinations, one combination of primers, GA(T,C)AT(T,C,A)TA(T,C)TA(T,C)GA(A,G)CA(T,C)GC for DIYYEHA and (T,C)TT(A,G)TC(A,G)TCCCA(A,G,T)AT(T,C)TT reversibly coding for KIWDDK, produced a 642-bp RT-PCR band. A cDNA library was constructed from mRNA of heat-shocked myxoamoebae using cDNA synthesis system plus (Amersham) and cDNA cloning system λgt10 (Amersham) according to the manufacturer's instructions. Using the 642-bp RT-PCR fragment described above as hybridization probe, one positive clone was obtained from screening 3.7×10^4 plaques. This positive clone contained an insert of about 2 kb. The insert was subcloned in pUC18 and sequenced by the dideoxy method (*22*) using synthetic primers.

Expression of ORF Product in Escherichia coli—The cutting site of the single *Pma* CI restriction site, CACGTG, resides at Arg-17 of the ORF (see Fig. 6). The greater part of the ORF following the *Pma* CI cutting site was ligated to the *Sma*I site of pUC19 to produce a chimeric protein consisting of 16 amino acids of lacZ α peptide and 585 amino acids of the ORF product. The resulting plasmid, pUC19(ORF), was introduced in JM109. The chimeric protein was induced by IPTG and analyzed by Western blotting (*23*) using anti-p66 antibody.

RESULTS

Purification of p66—As previously reported (*13*), p66 was expressed almost exclusively in heat-treated myxoamoebae, *Physarum* haploid cells (Fig. 1, lane 4). Very weak expression of p66 was observed in untreated myxoamoebae (Fig. 1, lane 3) or in untreated plasmodia, *Physarum* diploid cells (Fig. 1, lane 1). Heat-treatment did not cause any induction of p66 in plasmodia (Fig. 1, lane 2). Therefore, we purified p66 to homogeneity from heat-treated (40°C, 30 min) myxoamoebae, as described in "MATERIALS AND METHODS" (Fig. 2A, lane 2). This single

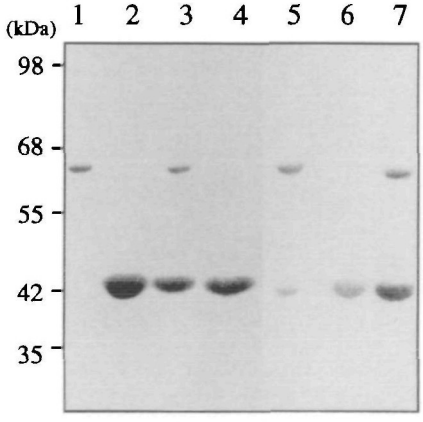

Fig. 3. **Co-precipitation experiments with purified p66.** The purified p66 or partially purified p66 fraction containing 42-kDa protein was mixed with the reaction mixture containing the purified *Physarum* actin and incubated. The mixture was then centrifuged at 100,000 × *g* for 2 h, and the supernatant and the pellet were analyzed by SDS-PAGE (CBB staining). Lane 1: The purified p66. Lane 2: The purified *Physarum* actin. Lane 3: Supernatant of a co-precipitation experiment of p66 and actin. Lane 4: Pellet of the co-precipitation experiment of p66 and actin. Lane 5: Partially purified fraction containing p66 and 42 kDa protein. Lane 6: Supernatant of a co-precipitation experiment of p66, 42 kDa protein and actin. Lane 7: Pellet of the co-precipitation experiment of p66, 42 kDa protein and actin.

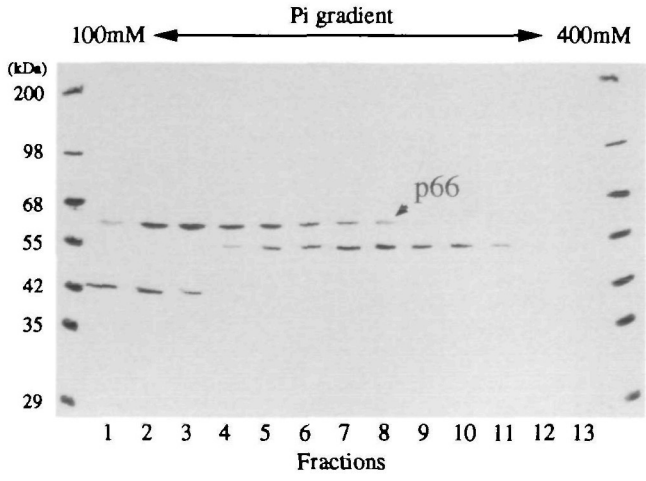

Fig. 4. **Chromatographic profile of p66 by hydroxylapatite.** Each fraction was analyzed by SDS-PAGE and stained with CBB.

peptide1 (K)NPNQADIYYEHAQPATVA
peptide2 (K)IWDDK
peptide3 (K)VLAVDNDK
T KA
S
peptide4 (K)XFASADQNRDIFVXD

Fig. 5. **Amino acid sequences of four lysyl-C peptides derived from the purified p66.** Two amino acid sequences underlined were selected and used for synthesis of the primers for RT-PCR.

band was recognized by the anti-p66 antibody (Fig. 2A, lane 4). The purified p66 bound to the ATP-agarose weakly and was eluted with 3 mM ATP (data not shown).

Interaction of p66 with Actin Filaments—When crude cell extracts of heat-shocked myxoamoebae were incubated with actin, five proteins with molecular mass of 90, 66, 42, 25, and 21 kDa were mainly precipitated together with polymerized actin (*13*). The 66-kDa protein reacted with anti-p66 antibody. To determine whether p66 binds actin filaments directly, the purified p66 was mixed with purified *Physarum* actin and incubated under actin-polymerization conditions. p66 was not co-precipitated with polymerized actin (Fig. 3, lanes 3 and 4), suggesting that purified p66 does not bind actin.

In the final step of purification, p66 was separated from two other proteins with molecular mass of 42 and 55 kDa by hydroxylapatite chromatography. Figure 4 shows the elution profile of the first hydroxylapatite column chromatography. The 42-kDa protein (p42) is not actin, because actin was separated from p66 at the first step of the purification and p42 did not interact with anti-actin antibody (data not shown). When a partially purified fraction (Fraction 2 of Fig. 4) containing both p66 and p42 was incubated with actin under actin-polymerization conditions, both p66 and p42 were precipitated with actin polymers (Fig. 3, lanes 5–7).

cDNA Cloning of p66—The purified p66 was digested with lysylendopeptidase, and the amino acid sequences of four derived peptides were determined (Fig. 5). A pair of oligonucleotide primers corresponding to the two determined sequences (underlined in Fig. 5) produced a 642-bp DNA fragment in a RT-PCR with mRNA from heat-treated myxoamoebae. This 642-bp DNA fragment contained a sequence coding for QPATVA following the primer sequence and proved to be a fragment of p66 cDNA (see Fig. 5, peptide 1). Using this 642-bp cDNA fragment as a hybridization probe, we screened 3.7×10^4 plaques of *Physarum* cDNA library and obtained one positive clone. This clone contained a 2-kb insert which has a 1,803-bp ORF corresponding to 601 amino acid residues and a poly(A) tail at its 3′ end (Fig. 6). A termination codon (TAA) occurs in frame just upstream of the first Met of the ORF. The calculated mass of this ORF product is 64,321 and is in good agreement with the apparent molecular mass of p66. To confirm that this ORF product is p66, the residues from Arg-17 to Asn-601 were expressed in *E. coli* as a chimeric protein (see "MATERIALS AND METHODS") and examined by Western blotting with anti-p66 antibody. The chimeric protein was indeed stained with anti-p66 antibody (Fig. 2B, lane 4).

p66 Is a Physarum Homologue of an Actin-Interacting Protein of S. cerevisiae—The predicted amino acid sequence of p66 has no homology with any conventional stress proteins. It contains the Walker motif A, GXXXXGKT, which is characteristic of a major class of nucleotide binding sites (*24*) at residues 213-220. This sequence may account

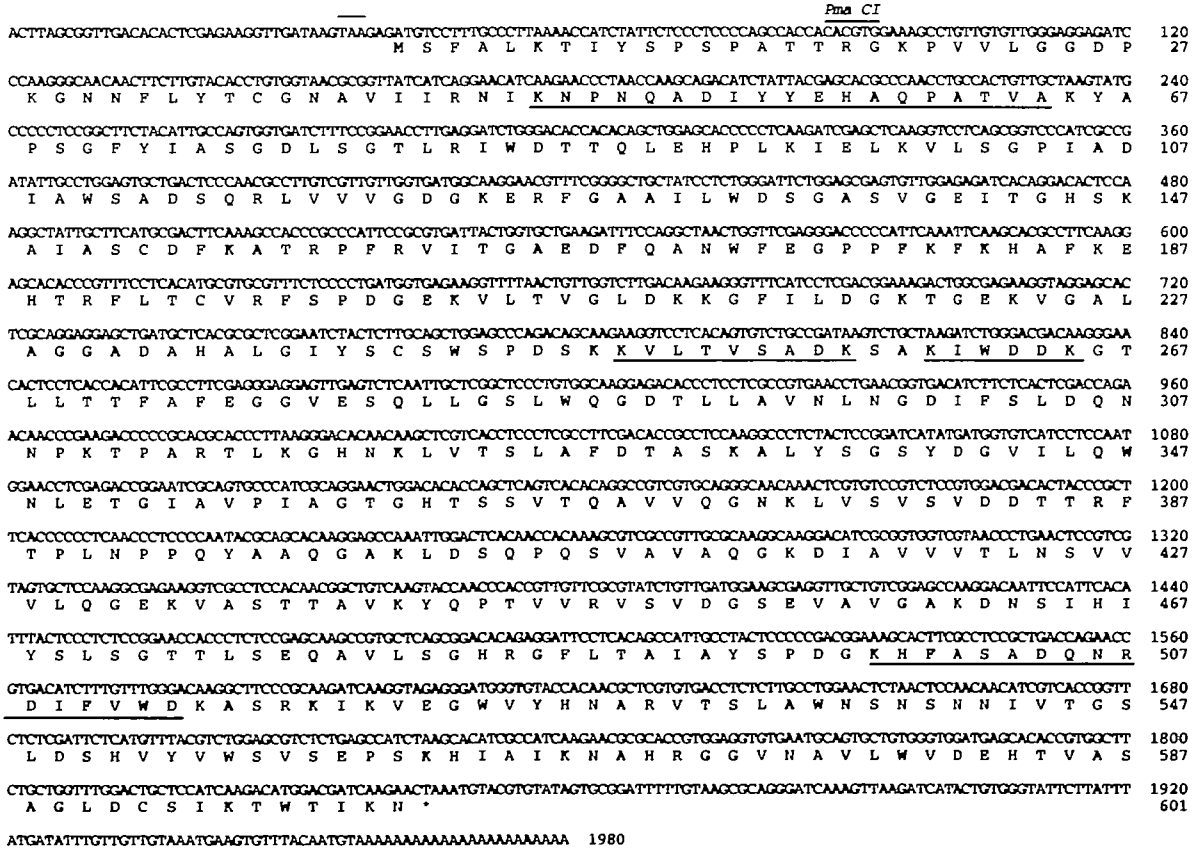

Fig. 6. **Nucleotide and predicted amino acid sequences of p66 cDNA.** The predicted coding region contains 601 amino acids (calculated mol wt=64,321, and pI=8.30). Peptide sequences derived from lysyl-C peptides of purified p66 are underlined. These sequence data are available from GenBank/EMBL/DDBJ under accession number U86011.

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p66   MSFA-LKTIYSPSPATTRGKPVVLGGDPKGNNFLYTCGNAVIIR-----NIKNPNQADIY
AIP1  MSSISLKEIIPPQPSTQRNFTTHLSYDPTTNAIAYPCGKSAFVRCLDDGDSKVPPVVQFT  60

p66   YEHAQPATVAKYAP--SGFYIASGDLSGTLRIW------DTTQLEHPLKIELKVLSGPIA
AIP1  GHGSSVVTTVKFSPIKGSQYLCSGDESGKVIVWGWTFDKESNSVEVNVKSEFQVLAGPIS  120

p66   DIAWSADSQRLVVVGDGKERFGAAILWDSGASVGEITGHSKAIASCDFKATRPFRVITGA
AIP1  DISWDFEGRRLCVVGEGRDNFGVFISWDSGNSLGEVSGHSQRINACHLKQSRPHRSMTVG  180

p66   EDFQANWFEGPPFKFKHAFKEHTR---FLTCVRFSPD-GEKVLTVGLDKKGFILDGKTGE
AIP1  DDGSVVFYQGPPFKFSASDRTHHKQGSFVRDVEFSPDSGEFVITVGSDRKISCFDGKSGE  240

p66   KVGALAGGADAHALGIYSCSWSPDSKKVLTVSADKSAKIWDDKGTL-LTTFAFE-GGVES
AIP1  FLKYIEDDQEPVQGGIFALSW-LDSQKFATVGADATIRVWDVTTSKCVQKWTLDKQQLGN  299

p66   QLLGSLWQGDT-LLAVNLNGDIFSLDQNNPKTPARTLKGHNKLVTSLAFDTASKALYSGS
AIP1  QQVGVVATGNGRIISLSLDG-TLNFYELGHDEVLKTISGHNKGITALTVN----PLISGS  354

p66   YDGVILQWNLETGIAVPIAGTGHTSSVTQAVVQGNKLVSVSVDDTTRFTPLNPPQYAAQG
AIP1  YDGRIMEW---SSSSMHQDHSNLIVSLDNS--KAQEYSSISWDDTLKVNGITKHEF----  405

p66   AKLDSQPQSVAVAQGKDIAVVVTLNSVVVLQGEKVASTTAVKYQPTVVRVSVDGSEVAVG
AIP1  ---GSQPKVASANNDGFTAVLTNDDDLLILQSFTGDIIKSVRLNSPGSAVSLSQNYVAVG  462

p66   -AKDNSIHIYSLSGTTLSEQAVLSGHRGFLTAIAYSPDGKHFASADQNRDIFVWDKASRK
AIP1  LEEGNTIQVFKLSDLEVSFD-LKTPLRAKPSYISISPSETYIAAGDVMGKILLYDLQSRE  521

p66   IKVEGWVYHNARVTSLAWNSNSNN----------IVTGSLDSHVYVWSVSEPSKHIAIKN
AIP1  VKTSRWAFHTSKINAISWKPAEKGANEEEIEEDLVATGSLDTNIFIYSVKRPMKIIKALN  581

p66   AHRGGVNAVLWVDEHTVASAGLDCSIKTWTIKN    601
AIP1  AHKDGVNNLLWETPSTLVSSGADACIKRWNVVLE   615
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Fig. 7. **Comparison of *Physarum* p66 amino acid sequence with the budding yeast AIP1.** Identical amino acid is indicated by (:). Similar amino acid is indicated by (.).

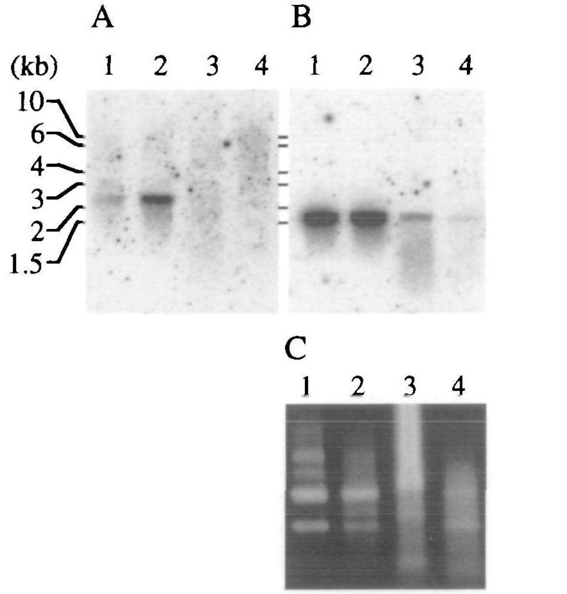

Fig. 8. **Stage-specific expression of p66 mRNA: Northern analysis.** Total RNA was denatured by glyoxal/dimethyl sulfoxide, run in a 1% agarose gel, and transferred to a nylon membrane filter. (A) The filter was probed with p66 cDNA. RNA size marker (Novagen Perfect RNA™ Markers, 0.2-10 kb) bands are indicated. (B) The same filter was stripped and reprobed with Ppa35 actin cDNA. (C) EtBr-staining of a conventional TBE agarose gel in which the same amount of RNA used in the blotting was run without denaturation. Lane 1: Myxoamoebae. Lane 2: Heat-treated myxoamoebae. Lane 3: Plasmodia. Lane 4: Heat-treated plasmodia.

for the weak ATP-binding activity of p66. We compared the amino acid sequence of p66 with entries in the data base and found that p66 is a *Physarum* homologue of a budding yeast protein, AIP1 (*25*). There was 31% identity of amino acids throughout the overall length between p66 and AIP1 (Fig. 7). AIP1 is a member of the WD-40 repeat protein family (*26*) and is considered to be an actin-interacting protein because of its interaction with actin in the two-hybrid system (*25*).

Stage-Specific Heat Shock Induction of p66 mRNA—mRNA expression level was examined by Northern blotting. p66 mRNA level was increased several-fold in heat-treated myxoamoebae (Fig. 8A, lane 2). Determination of RNA concentration in an RNA preparation from plasmodia by spectrophotometry was almost impossible because of the large amount of co-purified polysaccharide. However, from the fluorescence of ethidium bromide staining of the gel (Fig. 8C), it is obvious that, in plasmodia, only a faintly detectable amount of p66 mRNA was expressed (Fig. 8A, lane 3) and no heat-induction of p66 mRNA level was observed (Fig. 8A, lane 4).

DISCUSSION

In this study, we have purified a novel stress-induced protein, p66, from myxoamoebae of a true slime mold, *P. polycephalum*, and cloned p66 cDNA. Although p66 is a major product induced by heat shock in myxoamoebae of *P. polycephalum*, the predicted amino acid sequence of p66 showed no apparent homology with HSP70 or other conventional stress proteins. On the basis of sequence homology (31% identical), we concluded that p66 is a *Physarum* homologue of AIP1. *AIP1* has been isolated as a yeast gene encoding an actin-interacting protein, by the two-hybrid-protein interacting system (*25*). In the present study, the purified p66 did not have an actin-binding activity, although our previous work showed that p66 was co-sedimented with polymerized actin when crude cell extracts were incubated with actin under actin-polymerizing conditions (*13*). In the present study, p66 was co-precipitated with actin filaments in the experiment using partially purified p66 fraction. Recombinant yeast AIP1 synthesized and purified from *E. coli* has an activity to bind actin filaments (Matsumoto, S., unpublished observations). AIP1 protein of *Dictyostelium discoideum* shows not only an actin-binding activity but also other cofilin-like activities (Aizawa, H. *et al.*, submitted for publication). Furthermore, *AIP1* was independently isolated as a multi-copy suppressor of temperature-sensitive *cof1* mutation (Iida, K. *et al.*, submitted for publication). Therefore, it seems likely that p66 has an actin-binding activity. p66 may be a fragile protein that easily loses its actin-binding activity, or it may lose factor(s) required to maintain its actin-binding activity during the final step of purification. In *P. polycephalum*, p66 is predominantly induced in heat-shocked myxoamoebae, where it may function as a main modulator of actin filament organization, probably by exhibiting its cofilin-like activities.

Like AIP1, p66 is a member of the WD-40 repeat protein

family (for review, see Ref. *26*). Although the WD-40 repeat has been supposed to be involved in protein-protein interactions, the function of the WD-40 repeat in p66 remains to be elucidated. p66 contains the Walker motif A characteristic of a major class of ATP-binding sites (*24*). This is consistent with the observation that purified p66 has a weak ATP-binding activity (*13*).

As we have shown previously (*13*), the synthesis of p66 was induced when myxoamoebae of *P. polycephalum* were exposed to elevated temperatures. The induction of p66 by heat shock reflects an increase in p66 mRNA, suggesting that heat-shock-induced transcriptional control is involved in the induction. The finding that p66 is induced by heat shock in myxoamoebae but not in plasmodia was intriguing. Western blotting with anti-p66 antibody and pulse-labeling experiments in haploid or diploid cells of true slime revealed the induction of p66 only when haploid myxoamoebae formed microsysts as a result of stress (data not shown). The mechanism of such stage-specific transcriptional regulation depending upon heat shock remains to be elucidated.

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