Aginactin, an Agonist-regulated F-actin Capping Activity Is Associated with an Hsc70 in Dictyostelium*

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We have previously isolated an agonist-regulated actin filament capping activity, called aginactin, that is associated with a 70-kDa protein (Sauterer, R. A., Eddy, R. J., Hall, A. L., and Condeelis, J. S. (1991) J. Biol. Chem. 266, 24533–24539). A 2.0-kilobase clone isolated from a Dictyostelium λgt11 cDNA library screened with affinity-purified aginactin antibodies displays an overall sequence identity of 73% to the 70-kDa heat shock cognate protein, Hsc70, from various species. Aginactin capping activity and the 70-kDa protein bind to ATP-agarose columns and are quantitatively depleted from the load, indicating that an Hsc70 is associated with aginactin activity. Moderate stringency Southern blots indicate the presence of no fewer than six Hsc70-related sequences. Immunofluorescent staining of vegetative Dictyostelium AX3 cells with aginactin antibodies reveals a colocalization of aginactin-associated Hsc70 in F-actin-rich regions of the cell cortex and cell protrusions. Nuclei and organelles lacked positive staining indicating that the aginactin-associated Hsc70 is cytosolic. The levels of cytoskeletal-associated Hsc70 correlate with the loss of barbed end capping activity following cAMP stimulation, suggesting that the uncapping of barbed filament ends through an Hsc70-associated process may account for the increase in nucleation activity observed at 5 s following agonist stimulation.

Chemoattractant-induced increases in actin polymerization have been observed in a variety of cells including neutrophils (1, 2) macrophages (3), lung carcinoma cells (4, 5), and Dictyostelium amoebas (6, 7). In these diverse cell types, the underlying mechanism for increases in actin polymerization following chemoattractant stimulation appears to be conserved. In Dictyostelium, cell lysates show a cytoskeleton-sensitive actin nucleation activity that exhibits a transient increase within 5 s of stimulation with the chemoattractant, cAMP. Following low speed centrifugation of cell lysates, the nucleation activity is found associated with the F-actin containing cytoskeletal pellet suggesting that the nucleation activity is associated with the barbed ends of actin filaments. The nucleation activity in the cytoskeletal pellet is stable, however, addition of the lysate supernatant to the pellet causes a rapid loss of nucleation activity, suggesting an inhibitor of actin nucleation is present in the supernatant (7). Upon stimulation with the chemoattractant cAMP, the inhibitor is regulated with kinetics reciprocal to the nucleation activity, suggesting that dissociation of a capping protein from the barbed or preferred end of F-actin is responsible for the increase in nucleation activity following stimulation.

To distinguish the agonist-regulated capping activity from other capping activities in Dictyostelium, the following purification strategy was devised. Since the agonist-regulated capping activity is at a minimum 5 s after stimulation with cAMP, lysate supernatants from unstimulated cells and cells lysed 5 s post-stimulation were run on parallel columns. Only the peak of capping activity that showed a significant decline after cAMP stimulation was pooled and used as the starting material for the next column. Following sequential DE52 anion-exchange, hydrophobic interaction, fast protein liquid chromatography anion-exchange, and hydroxypatite chromatography, we have purified a 70-kDa protein associated with this cytosolic activity that inhibits actin nucleation and called it aginactin for AGonist-regulated INhibitor of ACTIN polymerization (8).

Aginactin is a barbed-end capping activity based on several criteria and is associated with a 70-kDa protein. Aginactin has an apparent $K_d$ for capping of 2.7 nM, neither nucleates nor severs F-actin and is Ca$^{2+}$ insensitive for all activities. In addition, the 70-kDa protein associated with aginactin can bind directly to actin filaments in a cosedimentation assay (8). In this paper, we present molecular genetic and biochemical evidence that the 70-kDa protein associated with aginactin is a heat shock cognate protein, Hsc70.

**MATERIALS AND METHODS**

*In Vitro Actin Capping Assay—*All polymerization assays were performed as described (7). Actin polymerization was monitored by the increase in fluorescence of pyrene-labeled actin in a mixture containing approximately 30% pyrene-labeled G-actin. The pyrene-labeled G-actin mixture was added to a final concentration of 2 μM in APAB (10 mM PIPES, pH 7.0, 50 mM KCl, 0.1 mM MgCl₂, 2.5 mM EGTA, 0.5 mM ATP, 1 mM dithiothreitol). For capping assays, fluorescence was monitored and actin polymerization was initiated 2–5 min later by the addition of 0.5 μM sheared F-actin seeds.

Protein Purification—Aginactin was isolated from Dictyostelium discoideum strain AX3 as described (8). ATP-agarose chromatography was performed as described (9) with slight modifications. The hydroxypatite pool containing active, purified aginactin was dialyzed against buffer D (20 mM Tris, pH 7.5, 20 mM NaCl, 0.1 mM EDTA, 3 mM MgCl₂, 1 mM dithiothreitol) and mixed with ATP-agarose resin equilibrated with buffer D. The mixture was then incubated at 4°C for 1 h with gentle agitation, poured into a 1 x 4-cm Diisco column. The column was sequentially washed with 2 column volumes each of buffer D, 0.75 M NaCl, buffer D and 1 mM GTP to elute any GTP.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) L22736.

1The abbreviations used are: PIPES, 1,4-piperazinediethanesulfonic acid; kb, kilobase(s); bp, base pair(s).
binding proteins. Bound Hsc70 was eluted with 3 mM ATP in buffer D, and fractions were analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting with aginatin antibodies.

Affinity Purification of Antibodies—A polyclonal antibody was raised against purified aginatin contained in the hydroxyapatite pool as described (8). The hydroxyapatite pool contained the aginatin-associated 70-kDa protein which was judged to be approximately 80% pure by Coomassie Blue staining of SDS gels and was the only major protein observed in the gels. Aginatin antibodies were affinity purified against the 70-kDa protein as described (10) with modifications. The hydroxyapatite aginatin pool was electrophoresed on a 10% SDS-polyacrylamide gel and Western blotted onto nitrocellulose (Schleicher & Schuell). The location of the 70 kDa protein was determined by staining of the nitrocellulose with 0.1% ponceau S in 1% acetic acid. The 70 kDa band was excised, blocked, and incubated with aginatin polyclonal antisera for 16 h at 4 °C with agitation. The pieces were washed with Tris-buffered saline (TBS), pH 7.5 (2 x 5 min), 1 M NaCl in TBS, pH 7.5 (1 x 5 min), and TBS, pH 7.5.

Antibodies were eluted with 0.1 M glycine-HCl, pH 2.8, 1 mM EGTA for 2 min, immediately neutralized with 1 M Tris, pH 8.5, dialyzed into TBS, pH 7.5, and used for incubations. Affinity purified aginatin antibodies recognize a single polypeptide band at 70 kDa in Western blots of Dictyostelium AX3 whole cells, ATP-agarose purified Dictyostelium Hsc70, and bovine brain Hsc70.

Protein Analysis—SDS-polyacrylamide gel electrophoresis was performed as described (11). For Western blots, proteins were transferred to 0.45 µm nitrocellulose membrane (Schleicher & Schuell) (12) and incubated with affinity purified aginatin antibodies, and processed and labeled with 35S-protein A as described (13). All autoradiographic images were scanned with an Ektro 1412 high resolution CCD camera, processed by Adobe Photoshop 2.5, and printed with a Sony Mavigraph Color Video Printer VP-500.

Isolation and Sequencing of cDNA Clones—A λgt11 cDNA library prepared against AX3 Dictyostelium discoideum amoebae (λ = 4) was kindly provided by P. Devreotes, Johns Hopkins University, and screened with affinity purified polyclonal aginatin antibodies labeled with 35S-protein A (ICN). Library screening and bacteriophage λ isolation were performed as described by Manniatis et al. (14). A λDNA was purified, digested and electrophoresed in 1% agarose TBE, pH 8.0. Inserts were then subcloned into pBLUESCRIPT II SK+ phagemid vector (Strategene) for sequencing. Sequencing reactions were performed using dye-dye chain termination method (Sequenase, Version 2.0, United States Biochemical Corp.) using T7, T3, and sequence-specific primers and confirmed by automated DNA sequencing (Applied Biosystems). For DNA and protein sequence analysis, Fasta and Gap programs included in the GCG-Wisconsin were used (15). To prepare for peptide microsequencing, purified aginatin-associated 70-kDa protein was dissolved in 8 M urea, 0.4 M NH4HCO3, reduced with 45 mM dithiothreitol, cysteine alkylated with 100 mM iodoacetamide, digested and electrophoresed for 16 h as described (16). Peptides were resolved on a Vydek C18 reverse-phase high performance liquid chromatography column using a trifluoroacetic acid-acetonitrile gradient. Selected tryptic peptides were subjected to Edman degradation and microsequenced at Harvard Microchemistry Facility, Harvard University.

Southern Blotting—Dictyostelium AX3 DNA was isolated from vegetative amoebae as described (17). Approximately 20 µg of DNA was digested with restriction enzymes, separated on 0.7% agarose in TBE, pH 8.0, and transferred onto ICN Biotrans Nylon membrane (14). 1.8 ng of DNA probe was labeled with [32P]dATP by random priming method (DirectPrime; AMBION) and hybridized overnight at 50 °C in hybridization solution (Worthington). Membranes were prehybridized for 20 min at 37 °C in 25 mM KPO4, pH 7.4, 5 × SSC, 5 × Denhardt’s solution, 50 µg/ml salmon sperm DNA, 50% formamide, 1% SDS. Hybridizations were performed for 14–16 h at 37 °C with the addition of 50% dextran sulfate to the hybridization buffer. Membranes were washed in 1 × SSC, 0.1% SDS at 45 °C (3 x 20 min). Southern blots were autoradiographed using Kodak X-Omat AR films with intensifying screens at −70 °C.

Immunofluorescence Microscopy—Dictyostelium AX3 cells were harvested and washed in PB (14.5 mM NaH2PO4, 5.2 mM KH2PO4, pH 6.6) and allowed to settle on ethanol-cleaned 12-mm circular coverslips (Fisher) for 30 min at 10 × 106 cells/ml. The cells were then overlaid with a 1.5% agarose sheet as described (18), but not allowed to flatten, fixed for 10 min in 2.5% formaldehyde (Baxter) in PB, and then in 3% PFA. Coverslips were washed briefly in PB, blocked for 15 min, and stained with 30 µg/ml affinity purified aginatin antibodies and 0.17 µM rhodamine phaloidin (Cappel) as described (19). Control antibody was prepared by incubating aginatin antibodies with 10-fold excess of purified 70-kDa protein immobilized on nitrocellulose for 16 h at 4 °C with gentle agitation. Fluorescein-labeled goat anti-rabbit IgG (Cappel) was prepared by preabsorption against formaldehyde fixed, acetone-extracted Dictyostelium AX3 cells (18), and used at 10 µg/ml. Cells were examined on a Bio-Rad MRC 600 confocal microscope equipped with a Kr/Ar laser to ensure complete separation of fluorescein and rhodamine channels. 1.5-µm optical sections of stained cells were imaged with a Nikon 60 × 1.4 numerical aperture flat field objective on a Nikon Diaphot. Prints were made using a Sony Mavigraph Color Video Printer VP-500.

RESULTS

Primary Structure of the 70-kDa Protein Associated with Aginatin—To obtain the primary sequence of the 70-kDa protein, we cloned the respective cDNA from a λgt11 library (λ = 4) prepared from the AX3 strain of D. discoideum. The library was screened with affinity purified antibodies raised against a highly purified hydroxyapatite pool of aginatin. A single clone of 2.0 kb, pAG-2.0, was isolated and sequenced. The pAG-2.0 cDNA has an overall length of 2,021 bp, contains an open reading frame of 1,908 bp coding for 636 amino acids, and terminates in a poly(A) tail (Figs. 1 and 2). Based on sequence comparison with other Hsc70s, pAG-2.0 lacks the first 24 nucleotides (8 amino acids) from the 5′ end, including the start AUG codon. Four additional positive clones were analyzed by EcoRI digestion and contained larger 5′ truncations than pAG-2.0 and were not characterized further. pAG-2.0 also terminates on a rare TGA codon. An analysis of codon usage in 56 Dictyostelium genes has shown that the TGA codon occurred with a frequency of 1/17,921 total codons (21). Comparison of the pAG-2.0 cDNA sequence with the GenEMBL data base using the FASTA program indicated the highest overall amino acid sequence identity (71–74%) was found between Hsc70 proteins from various eukaryotic species including human, rat, cow, and Drosophila. Fig. 3 shows a direct amino acid comparison between pAG-2.0 and Hsc70 from bovine brain and Drosophila using GAP program. Allowing for maximum alignment, aginatin displays an overall 74% identity, 84% similarity with bovine Hsc70 and 71% identity, 82% similarity with Drosophila Hsc70.

All Hsc70s thus far described are composed of two distinct domains. The ATPase domain which is contained in the N-terminal 450 amino acids is the most highly conserved domain among all Hsc70s (22). As expected, the percent identity of pAG-2.0 within the ATPase domain of bovine and Drosophila Hsc70 was higher at 80 and 79%, respectively. The more variable COOH-terminal 200 amino acids which contains the proposed substrate-binding domain was less conserved at 57.5 and 50%, respectively.

The deduced amino acid sequence of pAG-2.0 was confirmed by direct sequencing of peptides generated by tryptic cleavage of a purified pool of aginatin. Three separate internal tryptic peptide spanning a total of 30 amino acid residues
tide 90-312 of with three strongly hybridizing bands at 6.4, 6.2, and 3.3 kb identity to the highly conserved ATPase domain of various Hsc70 proteins. Of the eight restriction digests performed, 222-bp EcoRI/RsaI restriction fragment derived from nucleotide 271-481 was the greatest number of bands (Fig. 2). The probe fragment consisted of a sequence present in Dictyostelium AX3 cells, Southern blots were performed (Fig. 4). The probe fragment consisted of a single letter code below the nucleotide sequence. Amino acid sequence is numbered on the left, and the nucleotide sequence is on the right. Tryptic peptides derived from purified Hac70 associated with aginactin was subjected to microsequencing. Exact matches between selected tryptic peptides and the deduced amino acid sequence are underlined.

matched precisely the deduced amino acid sequence of pAG-2.0. In order to determine the number of Hsc70-related sequences present in Dictyostelium Hac70 derived from the cDNA clone pAG-2.0. The translated amino acid is shown in single letter code below the nucleotide sequence. Amino acid sequence is numbered on the left, and the nucleotide sequence is on the right. Tryptic peptides derived from purified Hsc70 associated with aginactin was subjected to microsequencing. Exact matches between selected tryptic peptides and the deduced amino acid sequence are underlined.

moderate stringency conditions, suggesting that Dictyostelium contains no fewer than six Hsc70-related sequences. Aginactin Binds to ATP-agarose Affinity Columns—One unique property of all Hsc70 proteins is the binding to ATP-agarose affinity columns and their elution with ATP (9). Since the deduced amino acid sequence of the pAG-2.0 and direct sequencing of tryptic peptides predicts that 70-kDa component of aginactin is an Hsc70, a hydroxyapatite pool of the unique property of all Hsc70 proteins is the binding to ATP-agarose affinity columns and their elution with ATP (9). Since the deduced amino acid sequence of the pAG-2.0 and direct sequencing of tryptic peptides predicts that 70-kDa component of aginactin is an Hsc70, a hydroxyapatite pool of the

FIG. 1. Structure of the pAG-2.0 cDNA clone. The relevant restriction sites are indicated: E, EcoRI; H, HindIII; N, NsiI; (R, RsaI probe only). Dotted line above pAG-2.0 indicate the location of the probe used for Southern blots. The arrows represent sequencing runs.
3 mM ATP (lanes 6 and 7). Capping assays performed on the ATP-agarose load and flow-through demonstrate that in the absence of ATP, approximately 63% of the aginactin capping activity is depleted from the load (Fig. 5A, shaded bar). However, in the presence of 0.4 mM ATP (open bar), only 2% of the capping activity is depleted from the load. Recovery of capping activity depleted from the ATP-agarose load was nonquantitative, with approximately 10-20% of the depleted capping activity recovered in the ATP eluent.

These results demonstrate that 1) antibodies prepared against aginactin recognize an Hsc70 and 2) the 70 kDa band and associated capping activity can bind to ATP-agarose and are specifically eluted with ATP suggesting that aginactin, an agonist-regulated, barbed-end capping activity, is associated with an Hsc70 in Dictyostelium. Based on these data and the high sequence identity with various Hsc70s, we conclude that the 70-kDa protein associated with aginactin is an Hsc70 of Dictyostelium.

Aginactin-associated Hsc70 Colocalizes with F-actin—Indirect immunofluorescence with affinity purified aginactin antibodies demonstrates that the aginactin associated Hsc70 is distributed throughout the cytoplasm and colocalized with the F-actin containing cytoskeleton of vegetative AX3 cells. Hsc70 staining of AX3 cells observed by rhodamine-phalloidin staining. Colocalization of Hsc70 with cortical F-actin cortex was observed at the leading edge by rhodamine-phalloidin staining. Colocalization of Hsc70 with cortical F-actin is particularly apparent in such cells. No Hsc70 staining of cell nuclei, organelles, or vesicles was observed in cells with any of these different morphologies.

Cytoskeletal Levels of Aginactin-associated Hsc70 Correlate with Decreases in Capping Activity—To quantitate changes in the level of cytoskeletal associated Hsc70, AX3 cells were starved for 6 h and lysed through a Nucleopore filter at various times following CAMP stimulation. A membrane-cytoskeletal pellet fraction was obtained from lysates by low speed centrifugation, Western blotted, and probed with aginactin anti-
Fig. 4. Southern blot analysis of Dictyostelium DNA probed with 5' Hsc70 sequences. 20 μg each of genomic DNA isolated from AX3 cells was digested with restriction enzymes, electrophoresed, transferred to nylon membrane, and hybridized with a 32P-labeled 222 bp EcoRI/RsaI fragment derived from the 5' end of pAG-2.0. Lanes 1, Xbol; 2, BglII; 3, CiaI; 4, NsiI; 5, BamHI; 6, EcoRI; 7, HindIII; 8, PstI. Lanes 1–4 and 5–8 are separate blots run under identical conditions. Molecular weight values are given in kb.

During the first 5 s post-stimulation, the level of Hsc70 associated with the cytoskeleton decreases, mimicking the decrease in the level of capping activity. This suggests that the uncapping of barbed filament ends upon stimulation leads to a decrease in cytoskeletal-associated Hsc70 and a corresponding increase in actin nucleation sites in the actin cytoskeleton as reported elsewhere (7).

Fig. 5. ATP-agarose chromatography of purified aginactin. Bar graph (panel A) shows depletion of aginactin capping activity in the ATP-agarose flow through as compared to the column load (shaded bar). In the presence of 0.4 mM ATP, the binding of capping activity to the column is significantly inhibited (open bar). A Western blot of a representative ATP-agarose column profile (panel B) shows binding of Hsc70 to the column, depletion from the load (compare lanes 1 and 2) and specific elution with ATP (lanes 6 and 7).

When cells are exposed to an external stress such as heat shock, they induce the synthesis of several classes of chaperone proteins that are thought to facilitate the stabilization and refolding of denatured proteins, assist in the proper assembly or disassembly of certain oligomeric protein complexes, and participate in maintaining precursor proteins destined for translocation across organelar membranes in an open, translocation-competent conformation (23). In recent years, the role of the 70-kDa heat shock protein class of chaperones has been intensively studied. This class includes forms that are strongly inducible by heat shock or other conditions of cellular stress (Hsp70) as well as forms which are constitutively expressed (Hsc70). Hsc70 (heat shock cognate 70) is a protein chaperone that assists in preventing premature or inappropriate folding of nascent polypeptides. Several Hsc70 proteins have been described that possess essential functions in the absence of stress. These include the yeast cytosolic Ssa proteins and the mammalian Hsc70, both of which can participate in the ATP-dependent disassembly of clathrin-coated vesicles. Organellar Hsp70s can also be found within the mitochondria (Ssc1p of yeast) and the endoplasmic reticulum (Kar2p in yeast and BiP in mammalian cells) (23).

In most eukaryotic organisms, Hsc70/Hsp70 proteins are members of multigene families. This complexity most likely reflects the involvement of certain members in stress response as well as constitutive and organellar functions. In Saccharomyces cerevisiae, eight distinct Hsp70-related genes have been characterized which function in a variety of cellular compartments including endoplasmic reticulum, mitochondria, nuclei, and cytosol (22, 23). Sequence analysis of the pAG-2.0 clone showed highest identity to the cytosolic Ssa1p and Ssa2p (72%) and less identity to Ssb1p, Kar2p, and Ssc1p (56.5, 58.6, and 48.6%, respectively) suggesting that the Hsc70 associated with aginactin is a cytosolic isoform. This is confirmed by the nonvesicular, cytosolic localization of Hsc70 by immunofluorescence in Dictyostelium. Southern blots of Dictyostelium DNA probed with sequence derived from the highly conserved NH2-terminal domain of pAG-2.0 clone suggest the presence of six Hsc70-related sequences, three of which show a strong hybridization with this probe under moderate stringency hybridization conditions.

A unique feature of Hsc70/Hsp70s is their ability to bind...
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FIG. 7. Correlation between cytoskeletal Hsc70 and levels of capping activity following chemostatic stimulation. Closed circles represent the levels of aginactin-associated Hsc70 present in the membrane-cytoskeletal pellet fraction of cell lysates following cAMP stimulation as measured by Western blotting with aginactin antibodies. Data shown were normalized for total cell protein. Normalization to cytoskeletal F-actin content gave similar results. The pretreatment level of Hsc70 is set at 100%. Closed squares represent the levels of capping activity (% inhibition) in cell lysate supernatants following cAMP stimulation as measured by pyrene-actin polymerization assay.

FIG. 6. Indirect immunofluorescent staining of aginactin-associated Hsc70 in Dictyostelium vegetative amoebas. Dictyostelium AX3 cells were prepared for immunofluorescence as described under “Methods and Materials” and stained with affinity-purified aginactin antibodies. Images are 1.5-nm optical sections generated by a Bio-Rad MRC600 scanning confocal microscope and therefore represent a constant pathlength. A, D, and G, phase; B and E, anti-agonactin; C, F, and I, F-actin. Note colocalization of Hsc70 with F-actin-rich regions such as the cell cortex (E and F). No staining was observed in control cells stained with anti-agonactin preabsorbed with ATP-agarose purified 70-kDa protein (H). Anti-agonactin staining of vesicles and nuclei was not found. Bar, 10 \textmu m.
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