Isolation and Characterization of a Regulated Form of Actin Depolymerizing Factor

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Abstract. Actin depolymerizing factor (ADF) is an 18.5-kD protein with pH-dependent reciprocal F-actin binding and severing/depolymerizing activities. We previously showed developing muscle down-regulates ADF (J. R. Bamburg and D. Bray. 1987. *J. Cell Biol.* 105: 2817–2825). To further study this process, we examined ADF expression in chick myocytes cultured in vitro. Surprisingly, ADF immunoreactivity increases during the first 7–10 d in culture. This increase is due to the presence of a new ADF species with higher relative molecular weight which reacts identically to brain ADF with antisera raised against either brain ADF or recombinant ADF. We have purified both ADF isoforms from myocytes and have shown by peptide mapping and partial sequence analysis that the new isoform is structurally related to ADF. Immunoprecipitation of both isoforms from extracts of cells prelabeled with [^{32}P]orthophosphate showed that the new isoform is radiolabeled, predominantly on a serine residue, and hence is called pADF. pADF can be converted into a form which comigrates with ADF on 1-D and 2-D gels by treatment with alkaline phosphatase. pADF has been quantified in a number of cells and tissues where it is present from ~18% to 150% of the amount of unphosphorylated ADF. pADF, unlike ADF, does not bind to G-actin, or affect the rate or extent of actin assembly. Four ubiquitous protein kinases failed to phosphorylate ADF in vitro suggesting that ADF phosphorylation in vivo is catalyzed by a more specific kinase. We conclude that the ability to regulate ADF activity is important to muscle development since myocytes have both pre- and posttranslational mechanisms for regulating ADF activity. The latter mechanism is apparently a general one for cell regulation of ADF activity.

TEMPORAL and spatial alterations in the actin cytoskeleton are necessary for numerous cellular functions. These changes in the assembly state and organization of the actin filaments are primarily due to the actions of actin-binding proteins (Stossel et al., 1985; Pollard and Cooper, 1986). The amounts and/or activities of these actin-binding proteins need to be regulated in order for the cell to change its actin cytoskeleton in response to environmental cues. A variety of regulatory mechanisms, including a direct response to levels of calcium and/or phospholipids (reviewed in Forscher, 1989), and modification by phosphorylation (Bahler and Greengard, 1987; Yamashiro et al., 1990) influence the activities of some actin-binding proteins.

Actin depolymerizing factor (ADF)[1], an 18.5-kD actin-binding protein is a constituent of a variety of tissues, cells, and cultured cell lines (Bamburg and Bray, 1987). After its initial discovery in embryonic chick brain (Bamburg et al., 1980), ADF was purified and characterized as a calcium-insensitive actin sequestering and severing protein with reciprocal pH-dependent F-actin–binding and –depolymerizing activities (Giuliano et al., 1988; Hayden et al., 1993; Hawkins et al., 1993). ADF has over 95% sequence identity with destrin, its mammalian homolog (Nishida et al., 1985; Adams et al., 1990; Abe et al., 1990; Moriyama et al., 1990), with which it shares similar biochemical activities (Hawkins et al., 1993). ADF also has >70% sequence homology to cofilin (Matsuzaki et al., 1988; Abe et al., 1990), a 19-kD actin-binding protein purified from several sources (Maekawa et al., 1984; Muneyuki et al., 1985; Abe and Obinata, 1989*a*) and widely distributed in a variety of vertebrate tissues and cultured cell lines (Yonezawa et al., 1987). The actin binding/depolymerizing activities of cofilin

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1. *Abbreviations used in this paper*: ADF, actin depolymerization factor; D, dimensional; MLCK, myosin light chain kinase; NEpHGE, nonequilibrium pH gradient gel electrophoresis; pADF, phosphorylated ADF; PKA, protein kinase A; PKC, protein kinase C; PVDF, polyvinyldifluoride; SPB, sample preparation buffer.

and ADF measured in vitro are nearly identical (Nishida et al., 1984; Yonezawa et al., 1985; Hayden et al., 1993; Hawkins et al., 1993). Since ADF and cofilin are often found in the same cell, including myocytes (Abe and Obinata, 1989b; Ono et al., 1993) and neurons (J. R. Jensen, M. DeWit, and J. R. Bamburg, manuscript submitted for publication), they may be considered isoforms generated by related genes. A protein with similar activity and >50% sequence homology to ADF has been isolated from amoeba (Cooper et al., 1986), and an ADF-related gene family (>40% sequence identity over 91 amino acids in the presumptive actin-binding domain) has recently been identified in plant pollen (Kim et al., 1993), making the ADF family of actin-binding proteins one of the most ubiquitous groups so far identified. The related protein in yeast is an essential component of the cortical actin cytoskeleton (Moon et al., 1993).

The amount and cellular distribution of ADF make it likely to be one of the major regulators of actin assembly in many cell types (Bamburg and Bray, 1987; Koffer et al., 1988; Abe and Obinata, 1989*a*). Immunofluorescence localization studies show that ADF is in higher concentration along the leading edge of ruffled membranes in cultured fibroblasts and in the growth cones of neurons (Bamburg and Bray, 1987; Jensen, J. R., M. Dewit, and J. R. Bamburg, manuscript submitted for publication). These locations are of significance because they are sites of actin polymerization and depolymerization associated with cell locomotion (Forscher and Smith, 1988; Theriot and Mitchison, 1992).

In developing skeletal muscle, ADF expression is down-regulated during and after myofibril assembly (Bamburg and Bray, 1987; Abe and Obinata, 1989*a*). To study the factors responsible for the down-regulation, we used myocyte cultures obtained from the dissociation of 10–11-d-old embryonic chick pectoral muscle. These cultured myocytes undergo fusion to form myotubes in which sarcomeres form and spontaneous contractions are observed. Unexpectedly, we discovered that the total amount of immunoreactive ADF increases during the 1 to 2 wk these cells are kept in culture. This increase is due to the formation of a new species of ADF which is not normally detected on immunoblots of muscle extracts unless the samples are heavily loaded. Here we report the isolation of this species, its characterization as an inactive form of ADF containing phosphoserine, and the identification of this posttranslationally modified ADF in a variety of tissues and cells. Parts of this manuscript have appeared in abstract form (Morgan, T. E., and J. R. Bamburg. 1988. *J. Cell Biol.* 107:466a; Lockerbie, R. O., and J. R. Bamburg. 1991. *J. Cell Biol.* 115:329a).

Materials and Methods

Cell Culture

Myocytes were prepared from dissected pectoral tissue of 10–11-d-old embryonic chick. The tissue was minced with a sterile scalpel, passed through a stainless steel mesh, and dissociated by trituration with a sterile glass Pasteur pipet in the culture medium. The cells were placed in a glass petri dish for 20 min during which time most fibroblasts attached to the substratum. Myocytes, which remain suspended in the medium, were decanted and plated in Primaria tissue culture dishes (Falcon, Oxnard, CA) at a density of 2×10^5 cells per 35 mm dish or 1×10^6 cells per 100 mm dish in DME containing 10% heat-inactivated horse serum, 2% chick embryo extract (prepared by the method of Bullaro, 1980), and 1× antibiotic–antimycotic solution. After 48 h in culture, 2 μg/ml cytosine arabinoside was added to inhibit fibroblast growth. Every third day half of the medium was removed and replaced with fresh medium. Cultured myocytes were photographed with a 20× phase contrast or Hoffman modulation contrast objective on a Nikon Diaphot microscope with Panatomic X film.

Murine myoblasts (C2C12 cells from Dr. Zach Hall, University of California, San Francisco, CA) were grown in 60 or 100 mm tissue culture dishes in growth medium (DME H-16 [low glucose], 20% FBS, 0.5% chick embryo extract, 2 mM L-glutamine, 1× antibiotic–antimycotic solution).

Chick embryo skin fibroblasts were prepared from the dorsal skin of 10-d-old embryonic chicks, dissociated with trypsin, and cultured on 60 mm tissue culture dishes in DME containing 10% FBS.

Growth Cone Particles

Growth Cone Particles (GCPs) were isolated from 18-d-old embryonic chick brain according to Pfenninger et al. (1983) with the modifications of Lockerbie et al. (1991). Verification of the embryonic chick brain GCP preparation was demonstrated by ultrastructural criteria and the enrichment of the developmentally regulated neuronal proteins, GAP-43 and $pp60^{src}$ (Lockerbie, 1990; Meiri et al., 1988).

Protein Purification, Determination, and Sequencing

Chick brain ADF was purified according to the method of Giuliano et al. (1988). Bacterially expressed ADF was prepared as described by Adams et al. (1990). ADF labeled with [^{35}S]methionine was prepared from 50 ml expression cultures induced with isopropylthio-β-D-galactoside in a methionine-free medium (methionine assay medium, Difco Laboratories, Detroit, MI) supplemented with 1 mCi [^{35}S]methionine. Skeletal muscle actin was purified from rabbit muscle acetone powder (Pardee and Spudich, 1982). After two cycles of polymerization and depolymerization, some of the actin was reacted with *N*-(1-pyrenyl) iodoacetamide and purified by chromatography following the procedure of Cooper et al. (1983). Solutions of G actin in 2 mM Tris, pH 7.5, 0.2 mM $CaCl_2$, 0.5 mM DTT, and 0.5 mM ATP were frozen in liquid nitrogen and stored at −70°C.

The purification of two forms of ADF from myocyte cultures is described in the Results section. Matrex gel Green A (Green A agarose) was obtained from Amicon Corp. (Danvers, MA). Hydroxylapatite was obtained from Bio-Rad Laboratories (Richmond, CA). Protease inhibitors were purchased from Sigma Immunochemicals (St. Louis, MO). Protease inhibition cocktail contained 2 mg/ml each of tosyl-arginine methyl ester, L-1-tosylamide-2-phenyl-ethylchloromethyl ketone, benzoyl-arginine methyl ester, and soybean trypsin inhibitor, and 0.2 mg/ml each of leupeptin, pepstatin, antipain, and chymostatin.

Concentrations of ADF and G actin were determined spectrophotometrically using $E^{1\%}$ at 280 nm = 6.45 (Giuliano et al., 1988) and $E^{1\%}$ at 290 nm = 6.3 (Houk and Ue, 1976), respectively. All other protein concentrations were determined by the filter paper dye-binding assay (Minamide and Bamburg, 1990) with ovalbumin as a standard.

Peptides from both ADF and pADF digests were sliced from polyvinyldifluoride (PVDF) membranes and sequenced on an ABI 471 automated protein sequencer in the Macromolecular Resource Facility, Colorado State University (Fort Collins, CO).

Sample Preparation

For polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE): freshly dissected tissues and/or cultured cells scraped from their dish after washing were prepared for SDS-PAGE by sonication (two 10-s pulses) in 20 vol of 10 mM Tris, pH 7.4, 2% SDS, 20 mM NaF, 10 mM DTT, 2 mM EGTA, and immediately placed in boiling water for 3 min. After cooling the samples on ice, proteins were precipitated (Wessel and Flügge, 1984) and dissolved in sample preparation buffer (SPB) containing 0.125 M Tris, pH 6.8, 1% SDS, 5% glycerol, 10% 2-mercaptoethanol, 0.01% bromophenol blue. For two-dimensional (2-D) gels: Samples for 2-D gels were prepared similarly except that the precipitated proteins were dissolved in lysis buffer (9.5 M urea, 2% NP-40, 2% ampholytes (pH 3–10) and 10% 2-mercaptoethanol) (O'Farrell, 1975).

For one dimensional (1-D) Peptide Mapping. Peptide mapping was performed according to the method of Plaxton and Moorhead (1989) on reduced and carboxyamidomethylated ADF and pADF. Proteins separated by SDS-PAGE were visualized by a brief staining with 0.125% Coomassie brilliant blue in 50% methanol, 10% acetic acid, and excised from the gel. To each gel slice in a microcentrifuge tube was added 420 μl of a reaction mixture, consisting of 25 mg BrCN (dissolved in 20 μl acetonitrile) in a total

of 420 μl of 0.3 N HCl. After incubating for 15 min at 37°C, the reaction mix was removed and each gel slice was equilibrated in 0.125 M Tris-Cl, pH 6.8, 0.1% SDS, 1 mM EDTA for 30 min at 30°C with six buffer changes.

Phosphoamino Acid Mapping. Immunoprecipitated ^{32}P-labeled ADF from GCPs and fibroblasts was dissolved in 6 N HCl (100 μl) sealed under nitrogen gas and incubated for 2 h at 110°C. The acid hydrolysates were repeatedly (three times) dried under vacuum in a Speed-Vac and resuspended in water.

Electrophoretic Methods

SDS-PAGE was performed by the method of Laemmli (1970) on 15% T, 2.7% C isocratic mini-slab gels (Idea Scientific, Minneapolis, MN). Prestained SDS-PAGE standards (18.5, 27.5, 32.5, 49.5, 80, and 106 kD) were from Bio-Rad Laboratories (Richmond, CA).

2-D gel electrophoresis was conducted using nonequilibrium pH gradient electrophoresis (NEpHGE) in the first dimension (O'Farrell et al., 1977; pH 3–10 ampholytes) in 75-mm long, 1.2-mm diam, tube gels. NEpHGE was run at 0.1 W per tube at 1,200 V maximum for 2 h. SDS-PAGE on mini-slab gels was used in the second dimension. Proteins were visualized by silver staining (Bamburg et al., 1991).

Electroblotting of proteins from 1- and 2-D gels onto nitrocelluose (0.1 μm; Schleicher & Schuell, Keene, NH) or PVDF membranes (Immobilon P; Millipore Corp., Bedford, MA) was performed as described previously (Bamburg and Bray, 1987) except that a Genie Blotter (Idea Scientific, Minneapolis, MN) was used for 1 h at 0.3 A. Some electroblots were stained for protein with colloidal gold (Moeremans et al., 1985).

Peptide Mapping. Gel slices containing BrCN-treated protein were transferred to the wells of a SDS–polyacrylamide gel (20% T, 0.5% C separating/10% T, 4.8% C stacking) containing 10% glycerol (Plaxton and Morehead, 1989). Electrophoresis was performed for 5.5 h at a constant current of 13 mA. Peptides were visualized in the gel by silver staining or were electroblotted onto PVDF membranes and visualized by Coomassie brilliant blue R staining.

Phosphoamino Acid Mapping. The aqueous hydrolysates were applied to a silica gel TLC plate (250-μm thickness; EM Reagents, Elmsford, NY) along with a mixture of phosphoamino acid standards. After electrophoresis for 60 min at 1,000 V in acetic acid/pyridine:water (50:5:945 by volume), standard amino acids were visualized with 0.3% ninhydrin in butanol. Autoradiography was used to visualize the position of the ^{32}P-labeled amino acid.

Immunological Methods

Antibodies. Purified bacterially expressed ADF (Adams et al., 1990) was used as the antigen to raise an immunoprecipitating rabbit antibody to ADF. The antiserum reacts specifically with ADF isoforms on 1- and 2-D immunoblots of chick brain extracts as reported for another ADF antiserum (Bamburg and Bray, 1987). An IgG fraction was prepared on protein G-agarose (Pierce Chemical Co., Rockford, IL) according to the standard protocol in the manufacturer's directions, and characterized for its ability to specifically immunoprecipitate ADF species (see Results). mAb (hybridoma supernatant) to chicken cofilin (mAb22) was a gift from Dr. Takashi Obinata (Chiba University, Chiba, Japan) (Abe et al., 1989).

Immunodetection on Immunoblots. The blocking and immunostaining of electroblots was performed as previously described (Bamburg et al., 1991). Blots were scanned on an HP scanner interfaced to a Microscan 2000 computer image analysis system (Technology Resources, Inc., Knoxville, TN). Internal standards of purified embryonic chick brain ADF (3–18 ng for nitrocellulose; 0.3–3 ng for PVDF) were included on gels used for quantitative measurements.

Immunoprecipitation. Cells from a single 60 mm tissue culture dish were lysed in 200 μl of 2% SDS, 10 mM Tris, pH 7.5, 10 mM NaF, 5 mM DTT, 2 mM EGTA, 2% BSA, and the lysate scraped from the culture dish and placed in a boiling water bath for 3 min. Then 24 μl of Triton X-100 was added, followed by 60 μl of buffer containing 100 mM Hepes, pH 7.4, 1.5 M NaCl, 10% Triton X-100. After adding 196 μl of water, 120 μl of a 2 mg/ml anti-ADF IgG fraction was added to give a final volume of 600 μl. This solution was mixed by rotation at 4°C for 1 h. Then 60 μl of a settled suspension of protein A-Sepharose beads, equilibrated in 10 mM Hepes, pH 7.4, 150 mM NaCl, 1% Triton X-100, was added. After mixing again on the rotating wheel for 1 h at 4°C, the Sepharose beads were sedimented in a microfuge and washed five times by resuspension in the equilibration buffer. Bound proteins were eluted from the beads in either SPB for 1-D gels or lysis buffer (O'Farrell et al., 1977) for 2-D gels.

Radiolabeling Methods

Myocyte Cultures. [^{32}P]Orthophosphate (0.1 mCi/ml) was added to the culture medium of 7-d myocyte cultures. After 48 h, the medium was removed and the cells washed with PBS (1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 2.7 mm KCl, 140 mM NaCl) at 37°C. Cells were scraped from the dish in 10 mM Tris-Cl, pH 7.4, 2 mM NaF, 1 mM NaN_3, 0.5 mM DTT, sonicated (2 × 10 s), and the cell homogenate centrifuged at 4°C for 30 min at 80,000 g_{ave}. The supernatant was applied to a column (0.5 × 1.0 cm) of DEAE-cellulose (DE-52; Whatman Corp., Clifton, NJ) equilibrated in the same buffer containing 50 mM NaCl. The flow-through was collected and concentrated on a CentriCell-20 (10 kD cut-off) (Polysciences Inc., Warrington, PA). The proteins were precipitated (Wessel and Flügge, 1984) and prepared for either SDS-PAGE by solubilization in 1× SPB or 2-D gel analysis by solubilization in lysis buffer.

C2 Cells. Cell monolayers (10^7 cells/60 mm dish) were rinsed with 150 mM NaCl, 10 mM Hepes, pH 7.4, 5.5 mM β-D-glucose, 5.0 mM KCl, 1.0 mM $MgSO_4$, 1.0 mM $CaCl_2$. The cells were covered with 2 ml of this same buffer and 1.0 mCi of [^{32}P]orthophosphate was added to each culture at a final phosphate concentration of 0.3 mM. After 90 min at 37°C, the radioactive medium was removed by aspiration and the cells washed twice with fresh buffer (5 ml). The cells from each dish were scraped into 300 μl of 10 mM Tris, pH 7.4, 2% SDS, 20 mM NaF, 10 mM DTT, 2 mM EGTA, sonicated 1 s and boiled for 5 min. Each cell extract was split into three aliquots and the proteins were precipitated (Wessel and Flügge, 1984). The pellets were solubilized in either 10 mM Tris, pH 7.4, 2% SDS, 10 mM DTT (for protein determination), SPB (for SDS-PAGE), or lysis buffer (for 2-D gel electrophoresis).

Chick Skin Fibroblasts. Fibroblasts in 60 mm culture dishes were labeled at 37°C for 3–4 h with 0.5–1.0 mCi [^{32}P]orthophosphate added in phosphate-free HBSS. Cells were washed with HBSS and lysed into 200 μl of 10 mM Tris, pH 7.4, 20 mM NaF, 2 mM EGTA, 0.5 mM DTT, 0.2% Triton X-100, and 10 μl/ml protease inhibition cocktail. These lysates were scraped from the plate, centrifuged for 1 min at 13,000 *g* in a microfuge, and the supernate used for the DNase I–Sepharose actin monomer binding assay described below.

GCPs. GCPs from embryonic chick brain were labeled with [^{32}P]orthophosphate at 1 μCi/μg protein for 90 min at 37°C in phosphate-free Krebs' buffer (Lockerbie et al., 1991). GCPs were lysed into 2% SDS in 10 mM Tris-Cl, pH 7.4, 20 mM NaF, 2 mM EGTA, 0.5 mM DTT, and 10 μl/ml protease inhibition cocktail for immunoprecipitation of ADF and pADF.

Autoradiography of dried gels and blots was performed on preflashed Kodak AR X-ray Film sandwiched between the gel or blot and a Dupont intensifying screen in a cassette stored at −70°C. Correct alignment of sample and film was achieved using phosphorescent GLOGO stickers (Stratagene).

In Vitro Phosphorylation/Dephosphorylation Assays

Myosin (1.2 mg/ml; 2μM), myosin light chains (1.2 mg/ml; 60 μM), and various concentrations of brain ADF (0.05, 0.10, 0.19, 0.29 mg/ml; 2.7 to 15.7 μM) were incubated for 30 min at 25°C with myosin light chain kinase (MLCK) (0.5 μg/ml) and 1 mM γ[^{32}P]-labeled ATP (10^4 dpm/pmole) in 25 mM Tris-Cl, pH 7.4, 4 mM $MgCl_2$ containing either 0.5 mM EGTA or 0.5 mM $CaCl_2$ with or without 0.5 mg/ml calmodulin. MLCK from chicken gizzard was a generous gift from Dr. John Kendrick-Jones (Medical Research Council Laboratory of Molecular Biology, Cambridge, UK). After stopping the reaction by the addition of 4× SPB (25% of volume) and incubating in a boiling water bath for 5 min, each sample was subjected to SDS-PAGE. Autoradiography was used to locate the labeled bands after silver staining and drying the gel.

Calcium–calmodulin-dependent protein kinase II (CaM kinase II) (McGuinness et al., 1985), and the catalytic fragment of protein kinase C (PKC) (Roth et al., 1989; Huang and Huang, 1986) both from rat brain, and protein kinase A (PKA) (catalytic subunit) from bovine heart (Beavo et al., 1974) were prepared according to published procedures. They were stored as 0.3 mg/ml stock solutions. Phosphorylation assays on chick ADF were performed using synapsin I as a positive control. ADF (200 pmoles) isolated from embryonic chick brain or myocyte cultures was incubated for 30 min at 30°C with 5.0 μg/ml of either rat brain CaM kinase II (in the presence of 30 μg/ml calmodulin and 0.3 mM calcium), or rat brain PKC catalytic subunit, or bovine heart PKA catalytic subunit in 50 mM Hepes, pH 7.4, 10 mM $MgCl_2$ including 50 mM γ[^{32}P]-labeled ATP (5 × 10^6 cpm/nmole). These conditions have been shown to be optimal for the labeling of synapsin I by CaM kinase II and PKA (Schiebler et al., 1986). The reaction was stopped by quick freezing the samples in liquid nitrogen. Sam-

ples were prepared for NEpHGE by addition of an equal volume of 2× lysis buffer (without urea) and solid urea to 9.5 M final concentration.

Dephosphorylation Assays. 1% SDS was added to the partially purified brain pADF and it was heated in a boiling water bath for 2 min, cooled, and the protein precipitated (Wessel and Flügge, 1984). The protein was redissolved in 100 μl 1% SDS in 50 mM Tris, pH 9.3, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$ and 1 mM spermidine. The sample was made 5% in Triton X-100, split into two portions, and 10 U of alkaline phosphatase (calf intestine) was added to one portion. Both the control and alkaline phosphatase–treated samples were incubated at 37°C for 1 h. The reaction was terminated in aliquots removed at zero time and at 60 min by precipitation of the protein (Wessel and Flügge, 1984). Precipitates were solubilized in SDS sample preparation buffer for 1-D gels or urea lysis buffer (O'Farrell et al., 1977) for 2-D gels.

Immunoprecipitated [^{32}P]-labeled pADF from either GCPs or fibroblasts was incubated with 4 U of alkaline phosphatase (calf intestine) in 100 mM Tris, pH 8.0, 200 mM glycine, 10 mM Zn acetate, 10 mM $MgCl_2$, 10 mM $CaCl_2$ at 37°C. At various times aliquots were removed and boiled in SDS sample preparation buffer. Samples were analyzed by SDS-PAGE, autoradiography, and densitometry of the autoradiograph.

ADF Activity Assays

DNase I Inhibition Assay. Actin depolymerizing activity was measured by the DNase I inhibition assay (Harris et al., 1982). Various concentrations of purified chick brain ADF, both forms of ADF isolated from myocyte extracts, and a buffer control were incubated with 5 μg F-actin for 15 min at room temperature. DNase I (2.5 μg) in 5 μl of 0.125 M Tris, pH 7.4, 5 mM $MgCl_2$, 2 mM $CaCl_2$, 1 mM NaN_3 at 4°C followed by DNA (90 μg in 900 μl of the same buffer) at 30°C, were added to each mixture and the increase in absorbance at 260 nm was followed at 30°C in a sipper cell of a Beckman DU-8B spectrophotometer. The maximal change in absorbance (ΔA_{260}) of each sample was compared to the control and plotted as percentage of DNase I activity remaining versus the amount of ADF (or pADF) used.

Pyrenyl-Actin Assembly. Pyrenyl-G-actin, incubated with 10 mM Tris, pH 7.4, 10 mM NaF, 0.5 mM DTT (control), or various concentrations of ADF or pADF from brain or myocyte extracts in this same buffer, was induced to assemble by the addition of KCl and $MgCl_2$ to 0.1 M and 2 mM, respectively. Fluorescence measurements were made in a spectrofluorometer (4800S Fluorometer; SLM-Aminco, Urbana, IL) using an excitation wavelength of 366 nm and an emission wavelength of 388 nm. Total sample volume was 100 μl. The relative fluorescence intensity was plotted against time.

Actin Monomer Binding Assay. Chick skin fibroblasts prelabeled with [^{32}P]orthophosphate were washed twice with PBS and extracted with 200 μl 10 mM Tris, pH 7.5, 2 mM EGTA, 20 mM NaF, 0.5 mM DTT, 10 μl/ml protease inhibition cocktail, and 0.2% Triton X-100. After centrifuging the extract for 3 min in a microfuge, the supernate was mixed for 1 min with either 60 μl of DNase I–Sepharose 4B slurry (50%) or Sepharose 4B alone. The resins were then pelleted, the supernate removed and saved (see below), and the pellet washed three times with 10 mM Tris, pH 7.5, 1 mM $MgCl_2$, 0.2% Triton X-100. The actin complexes bound to the resin were eluted by boiling in 2× SPB. pADF, and excess ADF that did not bind to the resin were immunoprecipitated from the supernate. Proteins in the actin complexes and immunoprecipitates were separated by SDS-PAGE, electroblotted onto nitrocellulose, and immunostained for ADF. The immunoblot was then subjected to autoradiographic analysis.

Results

One-Dimensional SDS-PAGE and Immunoblotting of Extracts from Myocyte Cultures Reveal Two Distinct ADF Species

Chick myocyte cultures at different stages of fusion and myotube development (Fig. 1) were extracted and the extracts subjected to 1-D SDS-PAGE and immunoblotting for ADF (Fig. 2). A new immunoreactive ADF species with a higher relative molecular weight than the ADF standard is visible by 2 d in culture and increases to approximately the same level as ADF by 10 d in culture. It remains at this level for at least a week. An immunoreactive form of ADF with similar mobility was also found in extracts of undifferentiated murine C2 cells. Quantitative densitometry of many of these immunoblots indicated that this second form of ADF was present as 45% of the total in 10 d myocyte cultures and 60% of the total in C2 cells. Total immunoreactive ADF (both forms) equals 0.1 ± 0.02% (SD) of the total protein in the myocyte extracts and 0.15 ± .03% of the total protein in C2 cell extracts.

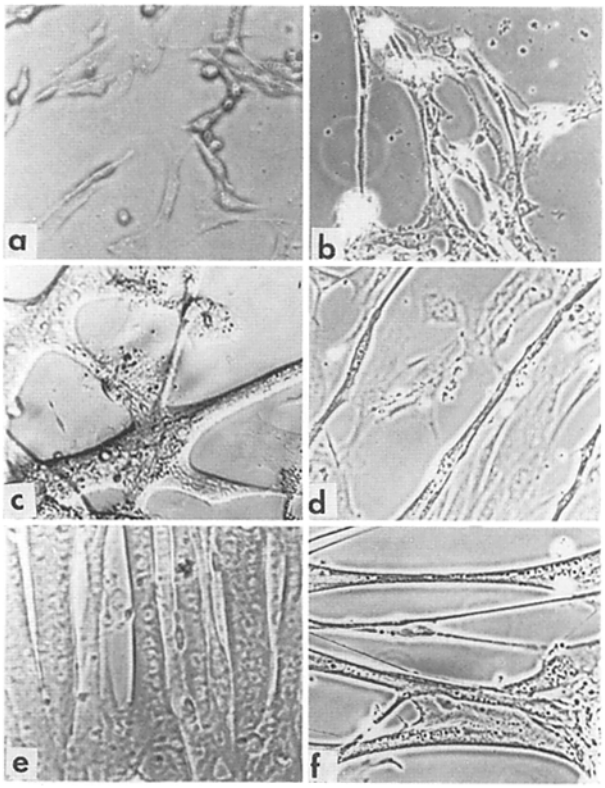

Figure 1. Phase contrast (*b*, *d*, and *f*) and Hoffman modulation contrast (*a*, *c*, and *e*) photomicrographs of chick embryo myocytes (E10) cultured for: (*a*) 1 d; (*b*) 2 d; (*c*) 3 d; (*d*) 4 d; (*e*) 5 d; (*f*) 6 d.

Two Forms of ADF from Myocyte Cultures Are Separated and Purified

Myocytes, cultured for 10 d, were washed with PBS, scraped from 40 100 mm culture dishes, and collected by low-speed centrifugation. Approximately 5 ml of packed cells were obtained. The cells were sonicated in 15 ml of 10 mM Tris, pH 7.4, 10 mM NaF, 0.5 mM DTT containing 150 μl of protease inhibition cocktail. This homogenate was centrifuged at 80,000 g_{ave} for 1 h. The supernate between the lipid layer and the pellet was applied to a DEAE–cellulose column (1.75 × 5.0 cm) equilibrated in the same buffer containing 50 mM NaCl but without the protease inhibitors. The flow-through (40 ml), containing all the immunoreactive ADF (both species), was collected and concentrated to 1–2 ml on Centriflo ultrafiltration membrane cones. No additional immunoreactive ADF could be detected in a high salt wash (600 mM NaCl) of the DEAE–cellulose column. The concentrate of the ADF-containing fraction (5 mg total protein) was applied to a Sephadex G-75 (2.5 × 90 cm) column equilibrated in 10 mM Tris, pH 7.4, 10 mM NaF, 0.5 mM DTT. Both im-

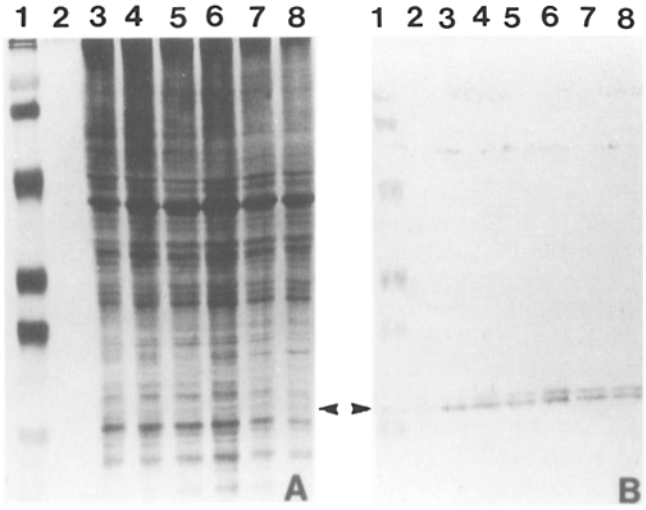

Figure 2. 1-D silver stained gel (*A*) and corresponding immunoblot (*B*) of extracts from myocytes cultured for various times. (Lane *1*) Prestained molecular weight markers; (lane *2*) ADF standard (5 ng); (lane *3*) 2 d culture; (lane *4*) 4 d culture; (lane *5*) 6 d culture; (lane *6*) 10 d culture; (lane *7*) 12 d culture; (lane *8*) 15 d culture.

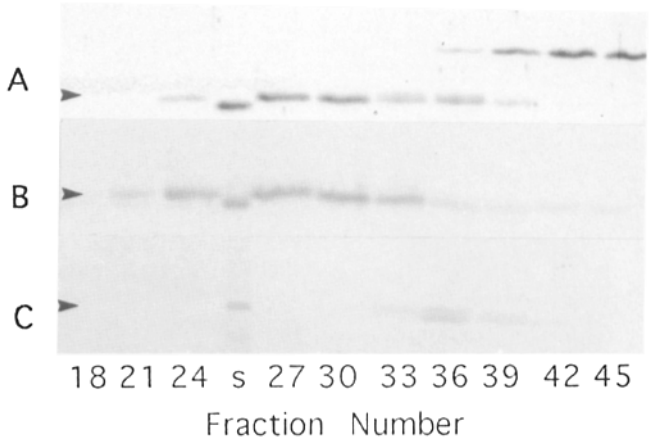

Figure 3. SDS-PAGE of fractions (0.6 ml) from hydroxylapatite chromatography of the green A-Sepaharose flow-through. (*A*) Silver stained gel; (*B*) immunoblots of similar gel stained for ADF; (*C*) immunoblot of similar gel stained for cofilin. Arrowhead marks position of identical mobility on each gel or blot. Lane s contains ADF standard in A and B, and C2 cell extract in C. Fractions containing pADF (21–32) were pooled and concentrated on a Centricon 10 microconcentrator to yield myocyte pADF.

munoreactive ADF species eluted at the trailing edge of the first major protein peak and were identified by SDS-PAGE and immunoblotting. The fractions (54–64 of 3-ml each) containing these forms of ADF were pooled and applied to a Green A agarose column (0.8 × 2.5 cm) equilibrated in the same buffer. One species of ADF (that with the higher relative molecular weight on SDS-PAGE) did not bind to the resin. As shown below, this form of ADF contains phosphate so it will be referred to as pADF. The pADF was collected in the flow-through (40 ml) and concentrated to 2–3 ml on a YM-5 ultrafiltration membrane under nitrogen pressure. The buffer was changed to 10 mM sodium phosphate, pH 7.4, 0.5 mM DTT by diluting (6×), and concentrating three times. This fraction was applied to a hydroxylapatite column (1.5 × 3 cm) equilibrated in the same buffer. After extensive washing with buffer (10 column volumes), the pADF was eluted with a 10 to 100 mM gradient (30 ml) of pH 7.4 sodium phosphate containing 0.5 mM DTT. The fractions containing pADF (Fig. 3 *B*) were largely resolved from fractions containing cofilin (Fig. 3 *C*), and were pooled and concentrated on a Centricon 10 microconcentrator.

The other species of ADF immunoreactive material, myocyte ADF, which bound to the Green A resin, was eluted with a 0–150 mM NaCl gradient (50 ml total) in the Green A column buffer. Fractions containing myocyte ADF were pooled and concentrated on a Centricon 10 microconcentrator. Both ADF and pADF were either directly assayed for activity or immediately frozen in liquid nitrogen. The confluent myotubes from forty 100 mm dishes yielded ~10 μg of pADF and 100 μg of ADF. The yield of pADF is 10× lower than the yield of ADF even though each form is present in approximately equal amounts in extracts from 7–10 d cultured myocytes. We have subsequently determined that loss of pADF occurs during concentration on the Centriflo ultrafiltration cones and on the Centricon 10. Fig. 4 shows a silver stained gel and immunoblot of each fraction obtained during this purification procedure.

Characterization of ADF and pADF Isolated from Myocyte Cultures

pADF and ADF Share Similar Antigenic Epitopes. Equal amounts of ADF and pADF were subjected to electrophoresis in SDS–polyacrylamide gels. Densitometric scans of Coomassie brilliant blue R–stained gels, colloidal gold stained electroblots, and ADF immunoblots were compared. ADF and pADF transfer and stain identically in each procedure, demonstrating that ADF and pADF have equal immunoreactivity to the antiserum. Amounts of sample loaded for each measurement were in the linear range for each detection method used. Identical immunoreactivty between the species was found using antisera made against electrophoretically pure ADF from either chick brain or bacterial culture

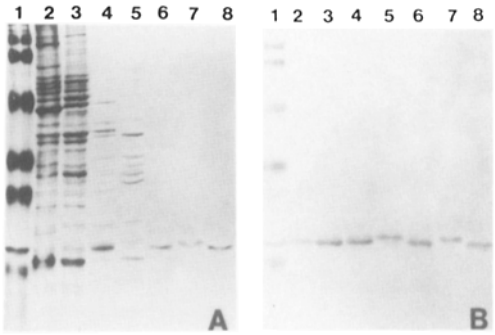

Figure 4. SDS-PAGE and immunoblot of aliquots taken during the purification of ADF and pADF from myocyte cultures. (*A*) Silver-stained gel and (*B*) corresponding immunoblot. (Lane *1*) ADF and molecular weight markers (17, 29, 36, 67, 94, 110 kD); (lane *2*) supernate of myocyte extract (3.3 μg); (lane *3*) DEAE-Cellulose flow-through (3.3 μg); (lane *4*) Sephadex G-75 pool (0.5 μg); (lane *5*) green A flow-through (0.2 μg); (lane *6*) ADF eluted from green A (40 ng); (lane *7*) pADF eluted from hydroxylapatite (40 ng); (lane *8*) brain ADF (40 ng).

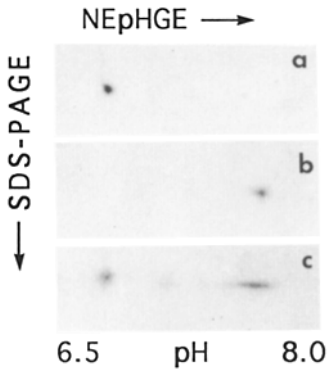

Figure 5. pADF is more acidic than ADF. Immunoblots of: (*a*) myocyte pADF; (*b*) myocyte ADF; and (*c*) mixture of both, separated on 2-D gels. About 250 ng of each protein were applied to the gels. ADF and pADF from myocytes each migrate identically to their counterparts from brain (not shown).

(recombinant ADF), substantially reducing the possibility that immuno cross-reactivity arises from a contaminating antigen in the ADF preparation.

pADF Is More Acidic Than ADF. The relative isoelectric points of ADF and pADF were compared on 2-D gels using NEpHGE in the first dimension and SDS-PAGE in the second (Fig. 5). Although proteins do not reach their actual isoelectric points during NEpHGE, protein standards of about the size of ADF (myoglobins) come very close to reaching their theoretical pI. Myocyte ADF comigrates with chick brain ADF at an approximate pI of 7.8. Myocyte pADF has an approximate pI of 6.6. This acidic shift of approximately 1.2 pH U is close to the theoretical shift in pI for the addition of a single phosphate group to ADF (calculated pIs = 7.42 and 6.35 for ADF and pADF, respectively, based upon the ADF amino acid sequence with its blocked NH_2-terminus (Adams et al., 1990) and the pKs of ionizable amino acids under denaturing conditions (Tanford, 1962).

pADF Is Not Cofilin or pCofilin. Extracts from the primary avian myocyte cultures were screened with both a mAb raised against chick cofilin (mAb22; Abe et al., 1989) and ADF antiserum. On 1-D immunoblots, mAb22 recognizes a single species in these extracts with a mobility very close to pADF (Fig. 3). However, on immunoblots of 2-D gels (Fig. 6 *A*), mAb22 reacts with two proteins, one migrating between pADF and ADF (~pI = 7.1), and the other more acidic than pADF (presumably phosphorylated cofilin), and not with either ADF or pADF. Extracts from murine C2 cells also contain two protein spots recognized by mAb22 (Fig. 6 *B*). The major immunoreactive spot migrates at pI of ~7.8 (almost directly above ADF), and corresponds to the "20-kD ADF" species previously identified in BHK cells (Koffer et al., 1988). Partial sequence analysis of this protein isolated from BHK cells has subsequently confirmed it to be cofilin (J. R. Jensen, L. S. Minamide, M. E. Adams and J. R. Bamburg, unpublished results). The other cofilin reactive spot in C2 cell extract has a pI of 6.2–6.3, corresponding to a phosphorylated form of cofilin previously identified in 3Y1 cells (Ohta et al., 1989). A second immunostaining of this blot with ADF antiserum localized separate species as ADF and pADF (Fig. 6 *B*). Reversal of the order of the antibodies gave similar results.

pADF and ADF Have nearly Identical Absorbance Spectra. The UV absorbance spectra of ADF and pADF isolated from cultured myocytes are nearly identical, eliminating consideration of several possible posttranslational modifications for the formation of pADF, such as ADP-ribosylation, which significantly alters the UV absorbance due to the addition of the purine nucleotide.

Peptide Maps of pADF and ADF Are Similar and Sequence of One Comparable Peptide Is Identical. Cyanogen bromide cleavage of ADF and pADF in gel slices was incomplete as both pADF and ADF gave bands of undigested protein (Fig. 7). However, a qualitatively similar pattern of peptides was obtained from the partial digests of both samples.

Corresponding peptides from the cyanogen bromide digests of the carboxamidomethylated ADF and pADF were excised from PVDF membrane and subjected to 12 cycles of automated protein sequencing. For both peptides the first cycle gave ambiguous results, but the sequence of the next 11 amino acids was confirmed to be identical. This peptide (VRKCSTPEEVK) runs from amino acid 20 to 30 in the ADF molecule. The similar peptide from chick cofilin has the sequence (VRKSSTPEEIK) (Abe et al., 1990). Thus, pADF is a modified form of ADF, and neither cofilin nor phospho-cofilin contaminates the purified pADF.

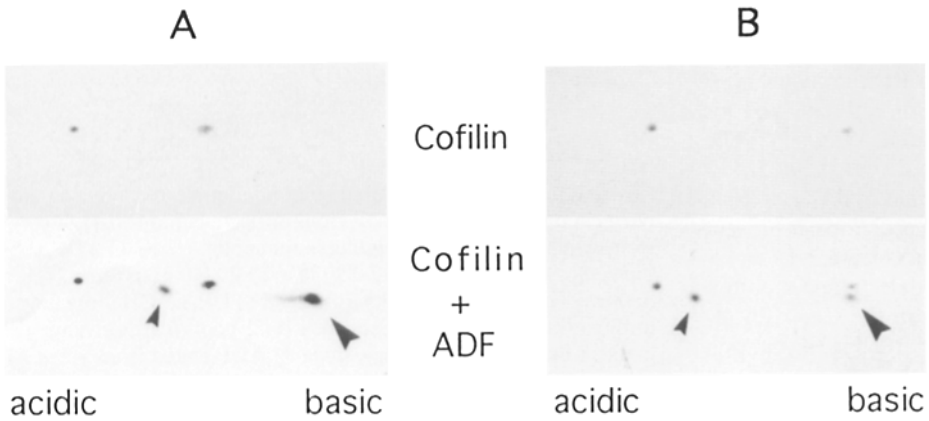

Figure 6. Cofilin and pADF are distinct proteins. Immunoblots of 2-D gels of extracts from (*a*) chick myocytes or (*b*) cultured mouse C2 cells (15 μg total protein) visualized first with mouse monoclonal antibody (mAb22) to cofilin (*top*) followed by rabbit antiserum to chick ADF (*bottom*). Reversing the order of staining blot (not shown) gave identical results except the proteins visualized during the first round of staining are more intense. Large arrowhead points to ADF and small arrowhead to pADF. The darkest staining cofilin species in the mouse C2 cell extract is identical to the "20-kD ADF species" previously isolated from BHK cells (Koffer et al., 1988; Bamburg et al., 1991).

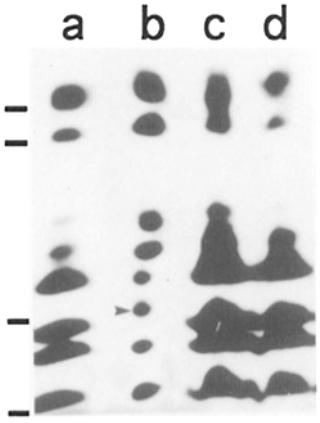

Figure 7. ADF and pADF have similar BrCN peptide maps on 1-D gels. Reduced and carboxamidomethylated brain ADF (*a*, 3 μg), myocyte pADF (*b*, 2 μg) and myocyte ADF (*c* and *d*, 6 and 4 μg) were digested in gel slices with BrCN and the resulting peptides separated on SDS-PAGE (20% T, 0.5% C). The upper band in each gel represents undigested protein (18.5 kD). Position of molecular weight markers: 18, 14.3, 6.2 and 3.4 kD. Sequence comparison was made for the peptide marked with an arrow and the corresponding peptide from brain digests.

Identification of pADF in Other Cells and Tissues

We have used 2-D gel immunoblotting to identify pADF and to quantify it relative to ADF in extracts of several cell lines, tissues, and subcellular fractions. Extracts were prepared in SDS buffer containing NaF, a phosphatase inhibitor, and were heated to boiling immediately after extraction to inactivate phosphatases and proteases. Table I shows that pADF is present in every sample in which we find ADF. Its level with respect to unphosphorylated ADF ranges from a low of 18% to >150%, or 15 to 61% of the total ADF (Table I).

Immunoprecipitation of ^{32}P-labeled pADF from Cell Extracts

The specificity of the ADF immunoprecipitation was evaluated using 2 μg [^{35}S]methionine-labeled recombinant ADF in the presence of 6 mg/ml BSA. An IgG fraction from preimmune serum did not immunoprecipitate ADF. The immunoprecipitation of ^{35}S-labeled ADF was completely inhibited by addition of a 100-fold excess of unlabeled ADF. Maximal precipitation of the ADF (1.2 μg) under the conditions used here is achieved with 240 μg IgG. Immunoprecipitates of extracts from chick skin fibroblasts prelabeled with [^{32}P]orthophosphate contain radiolabeled pADF as well as unlabeled ADF as shown by autoradiography of 2-D immunoblots (Fig. 8). Identical results were obtained with extracts from radiolabeled GCPs.

*Table I. pADF as a Percentage of Total ADF in Tissues and Cells**

	pADF as percent of total ADF ± SD
Tissue or tissue fraction	
Chick brain, 10-d-old embryo	21%
Chick brain GCPs	28 ± 5%
Chicken sciatic nerve, 6-wk old	27 ± 6%
Chick dorsal root ganglion, 8-d-old embryo	31%
Chick skin, 10-d-old embryo	16.5%
Chick muscle, 10-d-old embryo	21%
Primary Cell Cultures	
Chick skin fibroblasts, primary culture	51 ± 4%
Chick myocytes, 15-d-old culture	45%
Mouse macrophages, primary culture	15%
Cell Lines	
Rat PC12 cells, undifferentiated	47%
Mouse C2C12 cells, undifferentiated	61%
Mouse NIH 3T3 cells	38 ± 2%
Mouse WEHI-3 cells, suspension culture	18%

* Measured from densitometry of 2-D gel immunoblots. Standard deviation is given for samples on which multiple independent analyses were performed. All other results are averages of duplicates except for the WEHI-3 cells (single analysis).

Identification of Phosphoserine As the Major Phospho Amino Acid

Phosphoamino acid analyses of the immunoprecipitated pADF from ^{32}P-labeled GCPs (Fig. 9 *a*) and chick skin fibroblasts (data not shown) show that phosphoserine accounts for ~80% of the phosphorylated amino acid present with phosphothreonine accounting for the remaining 20%. No phosphotyrosine could be detected.

Conversion of pADF to ADF with Alkaline Phosphatase

Embryonic chick brain is a good source of pADF (Table I). We have used a modified purification procedure (eliminating the gel filtration step) to prepare partially purified pADF to see if it could be converted to ADF by alkaline phosphatase treatment. 2-D gel analysis of the partially purified material showed that the pADF is the only species with mobility in the 18–19-kD region. The zero time sample, 60-min incubated control and 60-min alkaline phosphatase–treated sample were run in duplicate on SDS-PAGE, one set being silver stained and the other electroblotted to PVDF membrane for immunodetection. The results (Fig. 9 *b*) clearly show that by 60 min >90% of the pADF has been converted to ADF. This alkaline phosphatase product of pADF digestion also comigrated with unphosphorylated ADF on 2-D gels (data not shown). In addition, the [^{32}P]phosphate on the pADF immunoprecipitated from both GCPs and chick fibroblasts is removed in a time-dependent manner by alka-

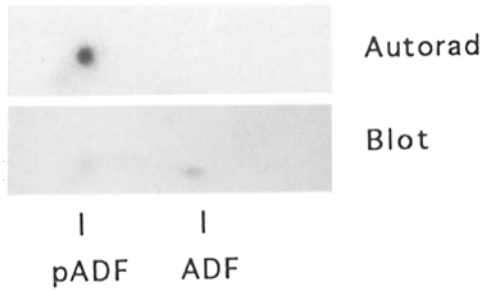

Figure 8. Only the pADF spot on 2-D gels of immunoprecipitates made from extracts of [^{32}P]orthophosphate-labeled cells contains radioactivity. Chick skin fibroblasts prelabeled for 4 h with [^{32}P]orthophosphate were washed, lysed in SDS buffer, and extracts were prepared for immunoprecipitation as described in Materials and Methods. The proteins were eluted from the protein A-Sepharose in lysis buffer and separated on 2-D gels. The immunoblot of the ADF region of the gel and its corresponding autoradiograph show that radioactivity is only associated with the pADF species.

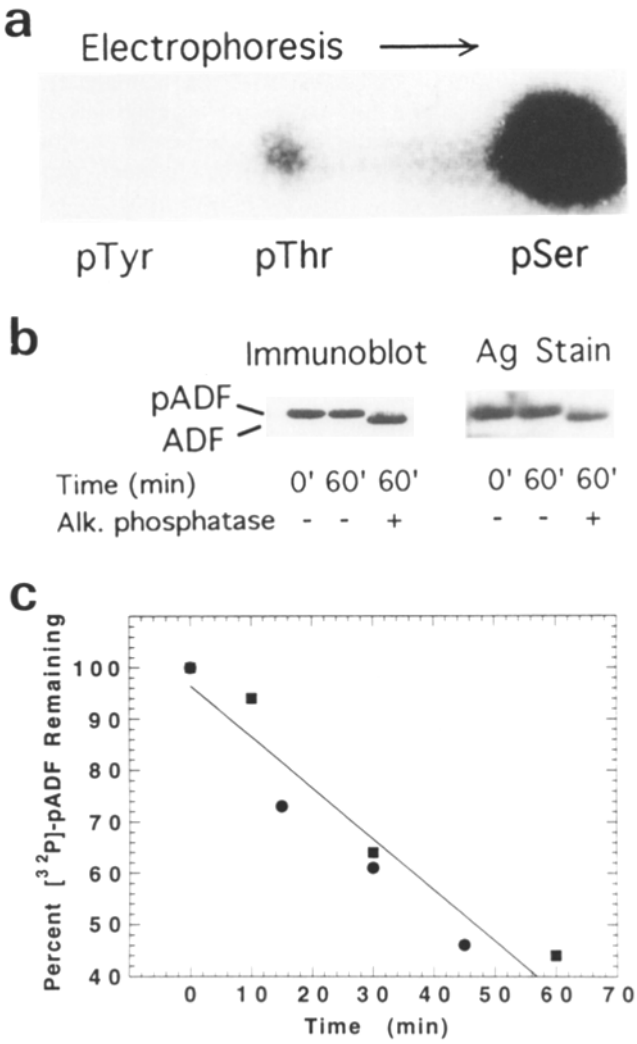

Figure 9. pADF contains phosphoserine and serves as a substrate for alkaline phosphatase. (*a*) Hydrolysates of immunoprecipitates from [^{32}P]orthophosphate-labeled GCPs (shown here) and chick skin fibroblasts, subjected to phosphoamino acid mapping, contained phosphoserine as the major phosphoamino acid. Densitometric scans of several autoradiographs showed that phosphoserine accounted for ~80% of the phosphoamino acid radioactivity, with the remainder being phosphothreonine. No phosphotyrosine was found in pADF. (*b*) Partially purified pADF (~5 μg of the pADF species) from 10-d-old embryonic chick brain, was digested with alkaline phosphatase as described in Materials and Methods. Aliquots were taken before addition of enzyme, 60 min after enzyme addition, and from a control sample incubated under identical conditions for 60 min without enzyme. Mobility shift of the alkaline phosphatase–treated material was also confirmed by 2-D gels. Greater than 90% of the pADF was converted to ADF in 60 min. (*c*) Immunoprecipitated pADF from chick skin fibroblasts (■) and GCPs (●) was resuspended and treated with alkaline phosphatase. The time course for the loss of [^{32}P]phosphate from the pADF shows a half-life of ~40 min under the conditions used.

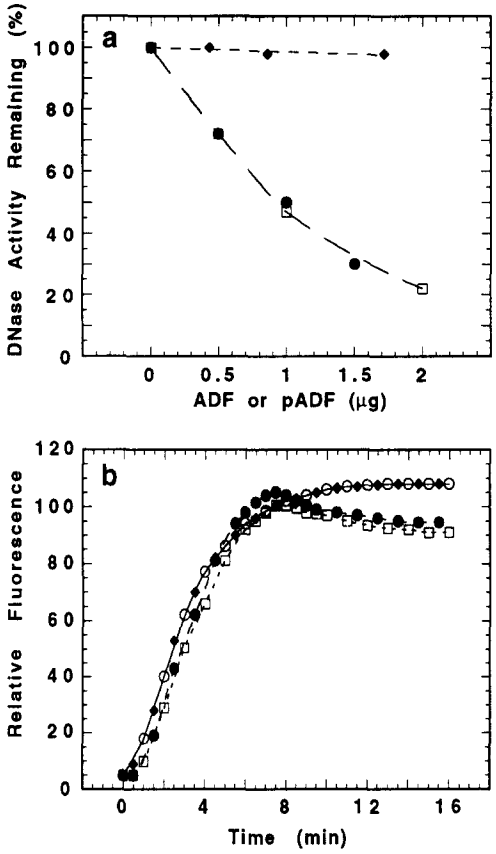

Figure 10. pADF is inactive in depolymerizing F-actin or altering the assembly of G-actin. (*a*) DNase I inhibition assay shows that brain (●) and myocyte (□) ADF can depolymerize F-actin, generating monomer which binds to and inhibits DNase I. Over the same concentration range, pADF (♦) is inactive. (*b*) The assembly kinetics of 5 μM pyrenyl-G-actin (○) are altered by the unphosphorylated forms of 0.4 μM myocyte ADF (□) and 0.5 μM brain ADF (●), but not by 5 μM pADF (♦). The delay in assembly is presumably due to the monomer sequestering ability of ADF while the slightly increased rate of assembly arises from severing of F-actin.

line phosphatase (Fig. 9 *c*), consistent with the results seen in the mobility shift.

pADF Is An Inactive Isoform of ADF

DNase Inhibition Assay. Purified chick brain ADF and ADF isolated from myocyte cultures generate similar amounts of monomeric actin from F-actin in this assay (Fig. 10 *a*). Myocyte pADF is completely inactive in generating actin monomer from F-actin under identical conditions.

Assembly of Pyrenyl-Actin. Chick brain ADF and myocyte ADF have nearly identical effects on the rate of assembly and the final extent of assembly of pyrenyl actin (Fig. 10 *b*). Myocyte pADF does not alter either the rate or final extent of assembly.

ADF and pADF Binding to Actin in Cell Extracts. Actin and actin-bound ADF in extracts of chick embryo fibroblasts prelabeled with [^{32}P]orthophosphate are co-sedimented by DNase I–Sepharose resin but not by Sepharose resin alone (Fig. 11 *a*). The ^{32}P-labeled pADF in these cell extracts remains in the supernate and is immunoprecipitated with IgG against ADF (Fig. 11 *b*). Therefore, pADF in cell extracts is not bound to actin that binds to the DNase I–Sepharose resin.

ADF Is Not a Substrate for Many Common Kinases

To determine if ADF can serve as a substrate for one of the known protein kinases, in vitro phosphorylation assays were

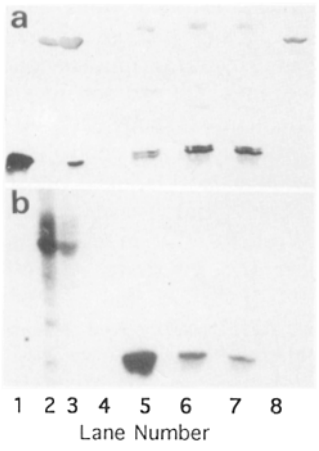

Figure 11. pADF in cell extracts is not bound to monomeric actin. DNase I-Sepharose resin was used to bind actin and actin monomer-binding proteins in two separate extracts prepared from [^{32}P]orthophosphate-labeled fibroblasts. The supernate remaining after DNase I-Sepharose treatment was used for immunoprecipitation of ADF. (*a*) Immunoblot, developed sequentially with ADF and actin antisera, and (*b*) corresponding autoradiograph. (Lane *1*) ADF standard; (lanes *2* and *3*) SDS-solubilized proteins binding to the DNase resin; (lane *4*) SDS-solubilized material binding to Sepharose resin without the covalently attached DNase; (lanes *5* and *6*) ADF immunoprecipitates from supernates of samples used for lanes *2* and *3*, respectively; (lane *7*) ADF immunoprecipitate from supernate of sample used for lane *4*; (lane *8*) actin standard. Although significant amounts of ADF were found in the DNase-Sepharose pellets (especially sample in lane *3*), no significant amount of radioactivity was associated with the ADF in this fraction. The pADF remained in the supernate and was immunoprecipitated (along with remaining unphosphorylated ADF).

used using ADF purified from embryonic chick brain. Kinases tested included: CaM kinase II, PKC, PKA, and MLCK. 1-D SDS-PAGE, immunoblotting, and autoradiography were used to detect the incorporation of radioactive phosphate into ADF. Under the experimental conditions used, MLCK phosphorylated myosin light chain alone or as part of the myosin complex, but did not phosphorylate ADF. Incubation of ADF with the catalytic subunit of PKA, PKC, or the holo enzyme of CaM kinase II also resulted in <0.25% of the ADF becoming labeled over 30 min, a time period over which the synapsin I control was stoichiometrically phosphorylated. The maximum amount of phosphate incorporated into ADF was catalyzed by CaM kinase II but only amounted to 0.0025 moles phosphate/mole of ADF. However, the ADF which is phosphorylated in vitro by CaM kinase II migrated identically on 2-D gels to the isolated myocyte pADF and was dephosphorylated when incubated with alkaline phosphatase.

Discussion

pADF Is a Regulated Form of ADF Produced by a Cellular Kinase

We have demonstrated here that a fraction of ADF in cells is phosphorylated, most likely on a serine residue. The pADF so produced is unable to depolymerize actin or bind actin monomers. It is unlikely that this inactivation occurs as a result of the additional hydroxylapatite chromatography step in the purification procedure since active ADF has been recovered from hydroxylapatite chromatography (Koffer et al., 1988). In addition, the pADF in freshly prepared cell extracts was shown not to bind to G actin. Thus, pADF represents a regulated form of ADF.

Production of pADF from ADF requires a protein kinase, the nature of which has yet to be identified. Our in vitro studies show ADF is not phosphorylated by smooth muscle type MLCK and ADF is not a substrate for either PKA or PKC and only a very weak substrate for CaM kinase II. However, pharmacological agents which perturb the activities of PKA, PKC, and CaM kinase II in both cultured cells and GCPs alter the phosphorylation of ADF (R. O. Lockerbie, unpublished results). Thus, ADF phosphorylation may be regulated by a kinase cascade under the control of transmembrane signals.

Cofilin and ADF Are Unique but Related Proteins Probably Regulated by a Common Mechanism

Cofilin, a protein which shares 70% sequence identity with ADF, exists in a phosphorylated state in cultured cells (Ohta et al., 1989). Since our ADF antiserum has a weak (3%) cross-reactivity with cofilin from BHK cells (Bamburg et al., 1991), we needed to conclusively demonstrate that pADF was not simply a phosphorylated form of cofilin. This was done using 2-D immunoblots developed with a mAb to cofilin that resolved the different cofilin isoforms, preceded or followed by development with a rabbit antiserum to ADF to resolve the position of ADF and pADF. Only the most acidic isoform of ADF or cofilin from either chick fibroblasts or mouse C2 cells, became radiolabeled when the cells were preincubated in [^{32}P]orthophosphate. The activity of p-cofilin has not been reported elsewhere, however it seems likely that phosphorylation of cofilin also inhibits its interaction with actin since no labeled cofilin in fibroblast extracts was found bound to the actin that bound to DNase I–Sepharose (Fig. 11). Thus, ADF and cofilin are isoforms which probably are regulated by similar posttranslational modifications.

Differentiating Myocytes have Alternative Mechanisms for Regulating ADF Activity

Muscle development in vivo is accompanied by a dramatic increase in actin levels and in actin assembly (Shimizu and Obinata, 1986). At embryonic day 10 in the chick ~40% of the actin is unassembled and this level declines to <2% by 2-wk posthatching. Over this same period the amount of total actin increases almost sevenfold, providing much of the new actin for assembling thin filaments. This increase in actin is accompanied by a 10-fold decrease in ADF from 0.2% of total protein to <0.02% (Bamburg and Bray, 1987) and a corresponding drop in ADF mRNA (Abe and Obinata, 1989*b*). During the same developmental period ADF and ADF mRNA levels remain high in brain (Bamburg and Bray, 1987; M. E. Adams, T. E. Morgan, and J. R. Bamburg, unpublished results). Thus, ADF expression is subject to tissue specific regulation.

Cultured myocytes mature into myotubes in vitro and undergo many of the same morphological changes as their in vivo counterparts, including cell fusion, the assembly of contractile sarcomeres, and an increase in total actin expression. However, these cultured myotubes do not fully mature, not progressing beyond the initial pattern of contractile protein isoform expression (Sutherland et al., 1993). The in vitro environment also does not provide sufficient information for developing myotubes to down-regulate the expression of the ADF gene. Immunofluorescence staining shows

that ADF is present and diffuse throughout the cultured myotubes, even after a week in culture (Abe and Obinata, 1989*a*). Muscle cells in culture cope with the additional ADF by converting it to the inactive pADF. Therefore, differentiating myocytes have alternative mechanisms for controlling ADF activity, suggesting that limiting ADF activity may be important for normal muscle development.

Potential Significance of ADF Phosphorylation in the Regulation of Actin Assembly in Other Cell Types

A recent study which used our ADF antisera showed that inhibitors of MLCK reduced the phosphorylation state of ADF and the regulatory myosin light chain in rat cortical astrocytes (Baorto et al., 1992). Of significance was the finding that the same inhibitors induced both process outgrowth and the breakdown of cortical F actin. A similar change in cell shape could be induced with dibutyryl cyclic AMP which also caused decreases in phosphorylation of myosin light chain and ADF, or by treatment with dihydrocytochalasin B which disrupts the cortical actin cytoskeleton. The authors suggest that regulation of ADF phosphorylation may be a major mechanism for controlling process formation in astrocytes. It also may be a general mechanism for controlling the disruption of actin filaments that has been shown to be necessary in several cases of cell morphogenesis including arborization of fibroblasts (Menko et al., 1983), elongation of retinal photoreceptor cells (Madreperla and Adler, 1989), and motility of neuronal growth cones (Forscher and Smith, 1988).

The generality of ADF phosphorylation as a regulatory mechanism is suggested by our finding of a significant amount of pADF in every cell and tissue type in which we find ADF. Why is limiting the amount of active ADF important to the cell when ADF levels in many cells are sufficient to sequester only a small amount of the monomeric actin pool? Other actin monomer-binding proteins, such as profilin (Carlsson et al., 1976) or thymosin β4 (Weber et al., 1992), sequester significant amounts of actin in many cell types. In resting polymorphonuclear leukocytes, thymosin β4 sequesters the majority of G actin. Chemoattractant stimulation results in actin assembly and release of the thymosin β4, but there is no change in the affinity for G actin of the thymosin β4 released (Cassimeris et al., 1992). Thus, the regulatory step must reside with another protein. The size of the unpolymerized pool of actin that these monomer sequestering proteins can maintain is determined by the affinity of the sequestering protein for the actin monomer and by the pool of unassembled actin with which they interact. This latter pool is commonly considered to consist of only the free actin monomer. However, in vitro studies to be presented elsewhere (J. R. Bamburg, S. M. Hayden, L. S. Minamide and P. Gunning, manuscript in preparation) show that the ADF-actin complex is also part of the profilin- and thymosin β4-reactive monomer pool. Therefore ADF can effectively increase the amount of actin depolymerized by a fixed amount of these proteins. Thus, in cells that contain only a few micromolar ADF and high concentrations (100 μM) of either thymosin β4 or profilin, the level of active ADF may actually regulate actin assembly by modulating the pool of monomer that can react with the sequestering protein.

At least two mechanisms for regulating the amount of active ADF in cells have now been demonstrated. Long-term regulation is achieved by the relatively slow process of down regulating ADF expression, probably by transcriptional control. This process occurs in tissues in which very low levels of actin remain unassembled (skeletal muscle and heart). Alternatively, the more rapid (and probably locally controlled) process of ADF inactivation by phosphorylation is more generally used by a wide variety of cell types. These findings are consistant with a fundamental role for ADF in regulating actin monomer pools in a number of cell systems.

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References

Abe, H., and T. Obinata. 1989*a*. An actin-depolymerizing protein in embryonic chicken skeletal muscle: purification and characterization. *J. Biochem. (Tokyo).* 106:172–180.

Abe, H., and T. Obinata. 1989*b*. Regulation of actin assembly in developing skeletal muscle: isolation of two actin regulatory proteins from embryonic chick skeletal muscle. *In* Cellular and Molecular Biology of Muscle Development. F. Stockdale and L. Kedes, editors. Alan R. Liss, New York. 197–206.

Abe, H., S. Ohshima, and T. Obinata. 1989. A cofilin-like protein is involved in the regulation of actin assembly in developing skeletal muscle. *J. Biochem. (Tokyo).* 106:696–702.

Abe, H., T. Endo, K. Yamamoto, and T. Obinata. 1990. Sequence of cDNA's encoding actin depolymerizing factor and cofilin of embryonic chicken skeletal muscle: two functionally distinct actin-regulatory proteins exhibit high structural homology. *Biochemistry.* 29:7420–7425.

Adams, M. E., L. S. Minamide, G. L. Duester, and J. R. Bamburg. 1990. Nucleotide seqence and expression of a cDNA encoding chick brain actin depolymerizing factor. *Biochemistry.* 29:7414–7420.

Bahler, M., and P. Greengard. 1987. Synapsin I bundles F-actin in a phosphorylation-dependent manner. *Nature (Lond.).* 326:704–707.

Bamburg, J. R., and D. Bray. 1987. Distribution and cellular localization of actin depolymerizing factor. *J. Cell Biol.* 105:2817–2825.

Bamburg, J. R., H. E. Harris, and A. G. Weeds. 1980. Partial purification and characterization of an actin depolymerizing factor from brain. *FEBS (Fed. Eur. Biochem Soc.) Lett.* 121:178–182.

Bamburg, J. R., L. S. Minamide, T. E. Morgan, S. M. Hayden, K. A. Giuliano, and A. Koffer. 1991. Purification and characterization of low-molecular weight actin-depolymerizing proteins from brain and cultured cells. *Methods Enzymol.* 196:125–140.

Baorto, D. M., W. Mellado, and M. L. Shelanski. 1992. Astrocyte process growth induction by actin breakdown. *J. Cell Biol.* 117:357–367.

Beavo, J. A., P. J. Bechtel, and E. G. Krebs. 1974. Preparation of homogeneous cyclic AMP-dependent protein kinase(s) and its subunits from rabbit skeletal muscle. *Methods Enzymol.* 38:299–308.

Bullaro, J. C. 1980. Monolayer cell cultures of embryonic chick skeletal muscle. *J. Tiss. Cult. Meth.* 6:91–95.

Carlsson, L., L. E. Nystrom, I. Sundkvist, F. Markey, and U. Lindberg. 1977. Actin polymerizability is influenced by profilin, a low molecular weight protein in non-muscle cells. *J. Mol. Biol.* 115:465–483.

Cassimeris, L., D. Safer, V. T. Nachmias, and S. H. Zigmond. 1992. Thymosin β4 sequesters the majority of G-actin in resting human polymorphonuclear leukocytes. *J. Cell Biol.* 119:1261–1270.

Cooper, J. A., S. B. Walker, and T. D. Pollard. 1983. Pyrene actin: documentation of the validity of a sensitive assay for actin polymerization. *J. Mus. Res. Cell Motil.* 4:253–262.

Cooper, J. A., J. D. Blum, R. C. Williams, Jr., and T. D. Pollard. 1986. Purification and characterization of actophorin, a new 15,000 dalton actin-binding protein from *Acanthamoeba castellanii. J. Biol. Chem.* 261: 477–485.

Forscher, P. 1989. Calcium and polyphosphoinositide control of cytoskeletal dynamics. *Trends Neurosci.* 12:468–474.

Forscher, P., and S. J. Smith. 1988. Actions of cytochalasins on the organiza-

tion of actin filaments and microtubules in a neuronal growth cone. *J. Cell Biol.* 107:1505–1516.

Giuliano, K. A., F. A. Khatib, S. M. Hayden, E. W. R. Daoud, M. E. Adams, D. A. Amorese, B. W. Bernstein, and J. R. Bamburg. 1988. Properties of purified actin depolymerizing factor from chick brain. *Biochemistry.* 27: 8931–8938.

Harris, H. E., J. R. Bamburg, B. W. Bernstein, and A. G. Weeds. 1982. The depolymerization of actin by specific proteins from plasma and brain: a quantitative assay. *Anal. Biochem.* 119:102–114.

Hawkins, M., B. Pope, S. K. Maciver, and A. G. Weeds. 1993. The interaction of human actin depolymerizing factor with actin is pH regulated. *Biochemistry.* In press.

Hayden, S. M., P. S. Miller, A. Brauweiler, and J. R. Bamburg. 1993. Analysis of the interactions of actin depolymerizing factor (ADF) with G- and F-actin. *Biochemistry.* In press.

Houk, T. W. J., and K. Ue. 1976. The measurement of actin concentration in solution. A comparison of methods. *Anal. Biochem.* 62:66–74.

Huang, K-P., and F. L. Huang. 1986. Conversion of protein kinase C from a Ca^{2+}-dependent to an independent form of phorbol ester-binding protein by digestion with trypsin. *Biochem. Biophys. Res. Commun.* 139:320–326.

Kim, S.-R., Y. Kim, and G. An. 1993. Molecular cloning and characterization of pollen-preferential gene encoding putative actin depolymerizing factor. *Plant Mol. Biol.* In press.

Koffer, A., A. J. Edgar, and J. R. Bamburg. 1988. Identification of two species of actin depolymerizing factor in cultures of BHK cells. *J. Muscle Res. Cell Motil.* 9:320–328.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–684.

Lockerbie, R. O. 1990. Biochemical pharmacology of isolated neuronal growth cones: implications for synaptogenesis. *Brain Res. Rev.* 15:145–165.

Lockerbie, R. O., V. E. Miller, and K. H. Pfenninger. 1991. Regulated plasmalemmal expansion in nerve growth cones. *J. Cell Biol.* 112:1215–1227.

Madreperla, S., and R. Adler. 1989. Opposing microtubule- and actin-dependent forces in the development and maintenance of structural polarity in retinal photoreceptors. *Dev. Biol.* 131:149–160.

Maekawa, S., E. Nishida, Y. Ohta, and H. Sakai. 1984. Isolation of low molecular weight actin-binding proteins from porcine brain. *J. Biochem. (Tokyo).* 95:377–385.

Matsuzaki, F., S. Matsumoto, I. Yahara, N. Yonezawa, E. Nishida, and H. Sakai. 1988. Cloning and characterization of porcine brain cofilin cDNA. *J. Biol. Chem.* 263:11564–11568.

McGuinness, T. L., Y. Lai, and P. Greengard. 1985. Ca^{2+}/calmodulin-dependent protein kinase II. *J. Biol. Chem.* 260:1696–1704.

Meiri, K. F., M. Willard, and M. I. Johnson. 1988. Distribution and phosphorylation of the growth-associated protein GAP-43 in regenerating sympathetic neurons in culture. *J. Neurosci.* 8:2571–2581.

Menko, A. S., Y. Toyama, D. Boettiger, and H. Holtzer. 1983. Altered cell spreading in cytochalasin B: a possible role for intermediate filaments. *Mol. Cell Biol.* 3:113–125.

Minamide, L. S., and J. R. Bamburg. 1990. A filter paper dye-binding assay for quantitative determination of protein without interference from reducing agents or detergents. *Anal. Biochem.* 190:66–70.

Moeremans, M., G. Daneels, and J. De Mey. 1985. Sensitive colloidal metal (gold or silver) staining of protein blots on nitrocellulose membranes. *Anal. Biochem.* 145:315–321.

Moon, A. L., P. A. Janmey, K. A. Louie, and D. G. Drubin. 1993. Cofilin is an essential component of the yeast cortical cytoskeleton. *J. Cell Biol.* 120:421–435.

Moriyama, K., E. Nishida, N. Yonezawa, H. Sakai, S. Matsumoto, K. Iida, and I. Yahara. 1990. Destrin, a mammalian actin-depolymerizing protein, is closely related to cofilin. *J. Biol. Chem.* 265:5768–5773.

Muneyuki, E., E. Nishida, K. Sutoh, and H. Sakai. 1985. Purification of cofilin, a 21,000 molecular weight actin-binding protein from porcine kidney and identification of the cofilin-binding site in the actin sequence. *J. Biochem. (Tokyo).* 97:563–568.

Nishida, E., S. Maekawa, E. Muneyuki, and H. Sakai. 1984. Action of a 19K protein from porcine brain on actin polymerization: a new functional class of actin-binding proteins. *J. Biochem. (Tokyo).* 95:387–398.

Nishida, E., E. Muneyuki, S. Maekawa, Y. Ohta, and H. Sakai. 1985. An actin-depolymerizing protein (destrin) from porcine kidney. Its action on F-actin containing or lacking tropomyosin. *Biochemistry.* 24:6624–6630.

O'Farrell, P. H. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250:4007–4021.

O'Farrell, P. Z., H. M. Goodman, and O'Farrell, P. H. 1977. High resolution two-dimensional electrophoresis of basic as well as acidic proteins. *Cell.* 12:1133–1141.

Ohta, Y., E. Nishida, H. Sakai, and E. Miyamoto. 1989. Dephosphorylation of cofilin accompanies heat shocked-induced nuclear accumulation of cofilin. *J. Biol. Chem.* 264:16143–16148.

Ono, S., H. Abe, R. Nagaoka, and T. Obinata. 1993. Colocalization of ADF and cofilin in intranuclear actin rods of cultured muscle cells. *J. Mus. Res. Cell Motil.* 14:195–204.

Pardee, J. D., and J. A. Spudich. 1982. Purification of muscle actin. *Methods Cell Biol.* 24:271–289.

Pfenninger, K. H., L. Ellis, M. P. Johnson, L. B. Friedman, and S. Somlo. 1983. Nerve growth cones isolated from fetal rat brain. Subcellular fractionation and characterization. *Cell.* 35:573–584.

Plaxton, W. C., and G. B. G. Morehead. 1989. Peptide mapping by CNBr fragmentation using a sodium dodecyl sulfate-polyacrylamide minigel system. *Anal. Biochem.* 178:391–393.

Pollard, T. D., and J. A. Cooper. 1986. Actin and actin-binding proteins. A critical evaluation of mechanisms and functions. *Annu. Rev. Biochem.* 55: 987–1035.

Roth, B. L., J. P. Mehegan, D. M. Jacobowitz, F. Robey, and M. J. Iadarola. 1989. Rat brain protein kinase C: purification, antibody production, and quantitation in discrete regions of hippocampus. *J. Neurochem.* 52:215–221.

Schiebler, W., R. Jahn, J.-P. Doucet, J. Rothlein, and P. Greengard. 1986. Characterization of synapsin I binding to small synaptic vesicles. *J. Biol. Chem.* 261:8383–8390.

Shimizu, N., and T. Obinata. 1986. Actin concentration and monomer-polymer ratio in developing chicken skeletal muscle. *J. Biochem. (Tokyo).* 99: 751–759.

Stossel, T. P., C. Chaponnier, R. M. Ezzell, J. H. Hartwig, P. A. Janmey, D. J. Kwiatkowski, S. E. Lind, D. B. Smith, F. S. Southwick, H. L. Yin, and K. S. Zaner. 1985. Nonmuscle actin-binding proteins. *Ann. Rev. Cell Biol.* 1:353–402.

Sutherland, C. J., K. A. Esser, V. L. Elsom, M. L. Gordon, and E. C. Hardeman. 1993. Identification of a program of contractile protein gene expression initiated upon skeletal muscle differentiation. *Dev. Dynamics.* 196:25–36.

Tanford, C. 1962. The interpretation of hydrogen ion titration curves of proteins. *Adv. Protein Chem.* 17:69–165.

Theriot, J. A., and T. J. Mitchison. 1992. Comparison of actin and cell surface dynamics in motile fibroblasts. *J. Cell Biol.* 118:367–377.

Weber, A., V. T. Nachmias, C. Pennise, M. Pring, and D. Safer. 1992. Interaction of thymosin $\beta 4$ with muscle and platelet actin: implications for actin sequestration in resting platelets. *Biochemistry.* 31:6179–6185.

Wessel, D., and U. I. Flügge. 1984. A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. *Anal. Biochem.* 138:141–143.

Yamashiro, S., Y. Yamakita, R. Ishikawa, and F. Matsumura. 1990. Mitosis specific phosphorylation causes 83kD, a nonmuscle caldesmon, to dissociate from microfilaments. *Nature (Lond.).* 344:675–678.

Yonezawa, N., E. Nishida, and H. Sakai. 1985. pH control of actin polymerization by cofilin. *J. Biol. Chem.* 27:14410–14412.

Yonezawa, N., E. Nishida, S. Koyasu, S. Maekawa, Y. Ohta, I. Yahara, and H. Sakai. 1987. Distribution among tissues and intracellular localization of cofilin, a 21 kDa actin-binding protein. *Cell Struct. Funct. (Japan).* 12: 443–452.