Distribution and Cellular Localization of Actin Depolymerizing Factor

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Abstract. Actin depolymerizing factor (ADF) is a low molecular mass (19 kD) protein that forms a tightly bound dimeric complex with actin. We have raised a rabbit antiserum to chick brain ADF and used it to analyze the distribution and cellular localization of ADF. We find that ADF is a major constituent of all chick embryonic and most adult tissues examined, accounting for 0.1–0.4% of the total protein. Some tissues have as much as 0.6 mol ADF per mole actin. Adult heart and skeletal muscle are unusual in having very low levels of ADF: <0.02% of the soluble protein. During the development of skeletal muscle, ADF levels are maximal up to ~11 d in ovo and then decline to reach their adult levels by 14 d posthatching. Brain tissue and cultured cell lines from several other vertebrates, including mammals, all possess proteins of identical size to ADF that are recognized by the ADF antiserum. No proteins are specifically recognized by the ADF antiserum in extracts from *Acanthamoeba castellanii* or from nerve tissue of several invertebrates.

Indirect immunofluorescence shows that ADF is present throughout the cytosol of most cells and at the leading edge of ruffled membranes and in the neuronal growth cone. Its abundance and widespread distribution together with its ability to sequester actin molecules, even those in an already polymerized state, suggest that ADF is a major factor in the regulation of actin filaments in many vertebrate cells.

As much as 50% of the actin in a typical animal tissue cell is in a nonfilamentous form (7, 17). The failure to polymerize is not an intrinsic property of the actin itself, since actins purified from most sources have similar properties to muscle actin (13, 33). Rather, it appears that specific proteins in the cytoplasm interact with actin to inhibit its assembly into filaments (reviewed in references 35 and 39). The best known protein of this kind is profilin (8), which is present in blood platelets in large amounts (14, 25) and has also been detected in other tissues such as thymus and brain (4, 28). Profilin binds to actin monomers and inhibits their assembly (8, 28), but appears to have little or no direct effect on F-actin (28, 36).

A second category of actin-binding protein has also been described that appears to affect the monomer–polymer equilibrium of actin. A monomeric protein, with a molecular mass of 19 kD, known as actin-depolymerizing factor (ADF),[1] was isolated from avian and mammalian brain (1, 2, 24). This protein binds to actin monomers and, unlike profilin, also severs existing actin filaments (29). Similar proteins have been found in starfish oocytes (23), porcine kidney (30), and *Acanthamoeba* (11). In view of the radical effects of ADF on actin in the test tube (1, 15, 30), it could play a central role in the regulation of actin filament assembly in the cell. However, before we can assess its importance, we need to know how much ADF there is in the cell, compared with actin, and how widely distributed this protein is in different tissues. These questions are addressed here.

1. *Abbreviations used in this paper*: ADF, actin depolymerizing factor; NEPHGE, non-equilibrium pH gradient gel electrophoresis.

Materials and Methods

Purification of ADF

ADF from 18-d embryonic chick brains was purified by the method of Bamburg et al. (1) as modified by Giuliano, K. A., F. A. Khatib, S. M. Hayden, E. W. Daoud, M. E. Adams, D. A. Amorese, B. W. Bernstein, and J. R. Bamburg (manuscript submitted for publication). Integration of a densitometer tracing of a Coomassie Blue–stained gel of purified ADF showed that >98% of the protein migrated as a single species of 19 kD. To ensure the highest purity obtainable for antibody production, ADF was subjected to electrophoresis on preparative SDS-containing polyacrylamide gels (15%), briefly stained with Coomassie Blue R, and the ADF band was excised. ADF was electroeluted from the gel by the method of Hunkapiller et al. (19). The ADF solution was freeze dried, the powder solubilized in 300 μl of water, and the SDS and dye were extracted from the protein (43). The precipitated protein was resolubilized in PBS (0.14 M NaCl, 8 mM phosphate, pH 7.2, 2.7 mM KCl).

ADF Activity Assay

Muscle actin was purified through two cycles of assembly using the method described by Pardee and Spudich (34). It was labeled with pyrenyliodoactamide by the method of Kouyama and Mihashi (20). ADF activity was quantitated by measuring its ability to lower the fluorescence of pyrenyl-F-actin. Fluorescence measurements were made in a spectrofluorometer (4800S; SLM-Aminco, Urbana, IL) in a microcell (3 mm × 3 mm) using a 100-μl sample volume. The amount of pyrene label on the actin was determined to be 10% (10).

Preparation of Rabbit Antiserum

Initial immunization of Dutch rabbits was performed with 150 μg ADF in complete Freund's adjuvant by multiple site, subcutaneous injections over axillary and inguinal lymph nodes. Booster injections (two) of 100 μg ADF in incomplete Freund's adjuvant were given intramuscularly at 4-wk intervals followed by an intravenous boost with 50 μg ADF in PBS. Rabbits were bled 10 d after the intravenous boost and serum was prepared by centrifugation of the clotted blood.

Gel Electrophoresis and Immunoblotting

SDS-PAGE was carried out according to Laemmli (21) in 0.5-mm-thick slab gels. Gels were stained with silver according to the method of Morrissey (27). Proteins were transferred electrophoretically from the gels to nitrocellulose (0.45 μm; Schleicher & Schuell, Dassel, FRG) for 3 h at 50 V (200–300 mA) using the buffer of Towbin et al. (40). Blots were either incubated in blocking buffer (0.1 g/ml nonfat dry milk, 10 mM Tris-Cl, 150 mM NaCl, pH 7.8) for 1 h and then washed for 1 h in 10 mM Tris-Cl, 150 mM NaCl, 0.05% Tween-20, pH 8.0 or were placed directly in the latter buffer. Unless the gels were heavily loaded with protein, the Tween-20–containing buffer prevented nonspecific binding of the antiserum to the nitrocellulose. After washing, the blots were incubated for 1 h in the primary antiserum (1:400 dilution of the rabbit antiserum in the above buffer containing 0.3% BSA). Blots were washed a second time in the same buffer, incubated for 1 h in the same buffer containing a 1:1,000 dilution of peroxidase-conjugated donkey anti-rabbit IgG (Amersham International, Amersham, UK), and washed again for 1 h in the same buffer before staining with diaminobenzidine (0.5 mg/ml) in 1% dimethylsulfoxide, 10 mM Tris, pH 7.5, 150 mM NaCl, 0.03% cobalt chloride, 0.02% hydrogen peroxide. Controls included substituting preimmune serum for the primary antiserum, eliminating the primary antiserum incubation, and eliminating the secondary antiserum incubation.

Quantitative reflectance densitometry of immunoblots was performed on a Chromoscan 3 (Joyce-Loebl, Gateshead, UK) using internal standards of ADF on each blot. Transmittance densitometry was performed on some blots after clearing with immersion oil (Cargille Laboratories, Cedar Grove, NJ; refractive index, 1.515) using a model DU 8B spectrophotometer (Beckman Instruments, Fullerton, CA).

Two-dimensional gel electrophoresis using non-equilibrium pH gradient gel electrophoresis (NEPHGE) in the first dimension was done as described by O'Farrell et al. (32) in 75-mm-long tube gels, 0.6-mm diam. Gels contained 2.5% pH 3–10 Servalytes (Serva Feinbiochemica, Heidelberg, FRG). Second dimension gels were 0.5-mm thick.

Sample Preparation

Tissue samples were prepared for SDS-PAGE by homogenizing the tissue in 20 vol of 2% SDS, 10 mM Tris, 0.5 mM dithiothreitol (DTT), pH 7.6 and boiling for 2 min. Protein determination was performed by Coomassie Blue G dye binding (5). Samples were diluted to 1–2.5 mg/ml protein in a final buffer containing 0.125 M Tris, pH 6.8, 5% glycerol, 1% SDS, 10% 2-mercaptoethanol, and 0.002% bromphenol blue for applying to gels. The unusually high concentration of 2-mercaptoethanol is necessary to prevent formation of ADF dimers and oligomers during electrophoresis.

Tissue samples for two-dimensional gel electrophoresis were prepared in 9.5 M urea, 2% NP-40, 2% ampholytes (pH 3–10), and 5% 2-mercaptoethanol (lysis buffer; 31).

Cell Culture

Dissociated cells from chick embryo dorsal root ganglia were prepared and cultured on glass (6). Chick embryo skin fibroblasts were prepared similarly from the back skin of 10-d embryos and were cultured on glass in Leibovitz L-15 medium (Gibco, Uxbridge, UK) supplemented with 10% FCS. Baby hamster kidney cells (BHK-21/C13) (9), Chinese hamster ovary (CHO) cells, and human fibrosarcoma (HT 1080) cells were grown on plastic in DME (Gibco) containing 10% FCS. Rat pheochromocytoma (PC 12) cells were grown on plastic in RPMI medium (Gibco) supplemented with 10% FCS.

Preparation of Frozen Sections

Pieces of cerebellum, intestine, and thigh muscle were removed from 10-d and 17-d chick embryos, fixed for 1–2 h in 4% paraformaldehyde in PBS, and prepared for sectioning in graded sucrose solutions (12). The tissues were frozen in Tissue-Tech (Miles Laboratories, Naperville, IL) and 10-μm sections were cut on a cryomicrotome. Tissue sections were adsorbed to slides pretreated with gelatin (12) and were incubated with 0.1 M glycine in PBS for 15 min before proceeding as described below.

Immunofluorescence Procedures

Cultured cells were fixed in one of five ways: (*a*) 0.25% glutaraldehyde in PBS containing 0.1% Triton X-100; (*b*) 0.25% glutaraldehyde in PBS followed by 0.1% Triton X-100 in PBS; (*c*) 0.1% Triton X-100 in PBS followed by 0.25% glutaraldehyde in PBS; (*d*) 4% paraformaldehyde in PBS containing 0.1% Triton X-100; (*e*) methanol at −20°C. After glutaraldehyde fixations, cells were treated with 2 mg/ml sodium borohydride in 50% methanol-50% PBS. All fixed cells and tissue sections were incubated in 10% donkey serum in PBS for 30 min before incubation with the primary antiserum (diluted into 1% BSA in 10 mM Tris, 150 mM NaCl, 0.05% Tween-20, pH 7.5). Before incubating with tissue sections, the primary antisera (pre- and postimmune) were adsorbed with adult chicken muscle acetone powder. The fixed cells and tissues were washed in PBS containing 0.1% Triton X-100 between antibody applications. Secondary antibodies included biotin-labeled donkey anti-rabbit IgG (Amersham) and fluorescein-labeled goat anti-rabbit IgG (Amersham) and were diluted for use into 10 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween-20. Rhodamine-phalloidin (Sigma Chemical Co. Ltd., Dorset, UK) and Texas red–streptavidin (Amersham) were diluted into PBS containing 0.3% BSA. After the final staining, cells or tissue sections were treated with a drop of Gelvatol (Cairn Chemicals, Ruislip, UK) containing 1,4-diazabicyclo(2.2.2)octane (Aldrich Chemical Co., Dorset, UK) as a photobleaching protectant. Light microscopy was performed with a Zeiss photomicroscope equipped with epifluorescence. Phase-contrast and fluorescence pictures were taken with Technical Pan and Tri-X films (Eastman Kodak Co., Rochester, NY), respectively, and were developed with HC-110.

Assay of Actin in Tissue Extracts

Samples of 12-d embryonic chick tissues weighing ~10 mg were sonicated (2 × 5 s) in 2 mM Tris, pH 7.5, 0.2 mM DTT, 0.1 mM ATP, 0.1 mM $CaCl_2$ so as to give a total protein concentration of 2 mg per ml. The solution resulting from the sonication of the tissue was stored on ice for 30–240 min before assaying for total actin by the DNase I inhibition assay (3). Briefly, an aliquot (10–100 μl) of the tissue extract was mixed with 50 μl of DNase I (50 μg/ml) and 0.9 ml of DNA (50 μg/ml) at 30°C was added. The sample was rapidly mixed and the change in absorbance at 260 nm was measured in a temperature-controlled cell of a Beckman DU-8B spectrophotometer. DNase I and DNA were prepared in 125 mM Tris, pH 7.5, 5 mM $MgCl_2$, 2 mM $CaCl_2$, 3 mM NaN_3. All tissue extracts were assayed within 1 h of preparation and again after 3 h. All but 3 of the 12 tissue extracts gave the same actin values to within 10% at the two times. In the other three tissues, values after 3 h were between 80 and 90% of the value obtained at 1 h. No correction for proteolysis of actin within the first hour was deemed necessary.

A standard curve for DNase I inhibition was prepared by incubating DNase I with G-actin (0.8–2.0 μg) prepared from rabbit muscle acetone powder (34). Its concentration was determined from its absorbance at 290 nm using $E^{1\%} = 6.3$ (18).

Results

Immunospecificity of the ADF Antibody

The immunospecificity of the rabbit antiserum to ADF was determined by immunoblot analysis of SDS-solubilized embryonic chick brain subjected to electrophoresis on both 5 and 15% polyacrylamide gels (Fig. 1). No bands developed on the nitrocellulose when preimmune serum was used for the primary antiserum bath, or when incubation with either the primary or secondary antiserum was omitted. The low percentage acrylamide gel was used to ensure transfer of high molecular mass proteins (up to 350 kD) whereas the higher percentage gel was used to resolve the proteins around the molecular mass of ADF (19 kD). Gradient gels did not give as good resolution in the ADF region as 15% isocratic gels. The only immunoreactive species observed on the gels when using dilutions of the antiserum from 1:400 to 1:4,000 co-

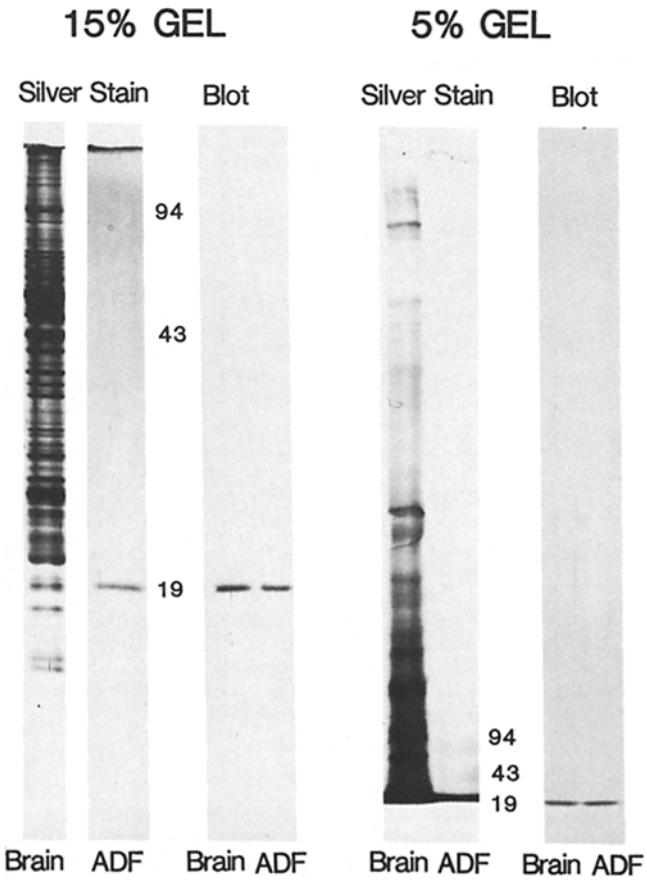

Figure 1. Immunoblot analysis of chick brain extract and ADF after electrophoresis on 5 and 15% acrylamide gels. Silver-stained gels contain 50 ng of purified ADF or 10 μg of protein from a 13-d embryonic chick brain homogenized directly into SDS-containing buffer. Gels for immunoblots contained 4 μg of chick brain protein or 10 ng of ADF. Immunoblots shown were incubated with antiserum to ADF; no bands developed on immunoblots incubated with preimmune serum at the same dilution.

migrated with the purified ADF standard. Therefore, the ADF immunoreactive species does not arise from a larger precursor molecule.

Two-dimensional gels and immunoblots of the chick brain protein (Fig. 2) demonstrated that the antiserum recognized two components of 19 kD in the brain extract. Both of these components are in the purified ADF and can form complexes with actin that are stable to gel filtration (Giuliano, K. A., F. A. Khatib, S. M. Hayden, E. W. Daoud, M. E. Adams, D. A. Amorese, B. W. Bernstein, and J. R. Bamburg, manuscript submitted for publication). Thus, the antiserum recognizes the two forms of ADF present in the preparations of purified protein. The ratios of isoforms of ADF obtained from densitometry of Coomassie Blue–stained two-dimensional gels and from immunoblots of purified ADF are both ~4:1; a similar ratio was found on immunoblots of extracts from whole brain (Fig. 2). Thus, each of these isoforms has similar reactivity with the antiserum.

The antiserum inhibits the ability of ADF to depolymerize pyrenyl-F-actin; this inhibitory effect can be overcome by addition of pure ADF. A titration was performed from which it was calculated that 1 μl of antiserum inactivates 3.52 μg of ADF.

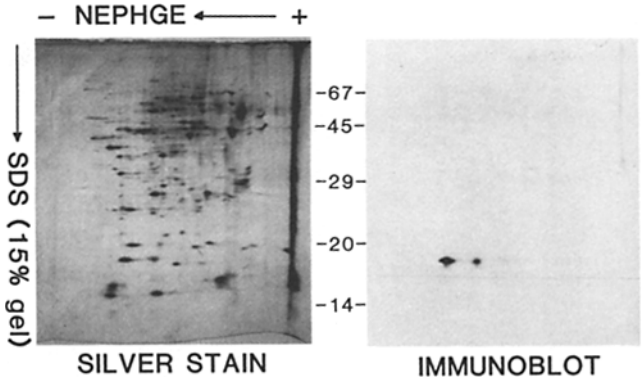

Figure 2. Immunoblot analysis of chick brain extract after electrophoresis on a two-dimensional gel. Samples applied to the NEPHGE tube gels for silver staining containing 15 μg of protein from a 13-d embryonic chick brain, whereas the gel for immunoblot analysis contained 10 μg. Tube gels were run for 2,000 Vh. Second dimension slab gels contained 15% acrylamide.

Tissue Distribution of ADF and Actin

The levels of ADF as a percentage of total protein were measured by densitometry of immunoblots of 12- and 13-d embryonic chick tissues (Table I). A single ADF species of 19 kD was observed on the immunoblots for all the embryonic tissues examined. The highest levels of ADF were found in the central nervous system whereas the lowest levels occurred in the liver and heart. Smooth and skeletal muscle tissues had intermediate levels.

Actin varied from 0.5 to 3.4% of the total protein in 12–13-d embryonic chick tissues (Table I). Note that at this stage in development, the actin levels in the nervous system and smooth muscle tissues were higher than in cardiac and skeletal muscle. The molar ratio of ADF to actin was lowest in heart and gizzard and highest in kidney. In all tissues, however, ADF was present in significant amounts when compared with actin.

Tissues from the adult chicken were not as readily solubilized in the SDS-containing buffer. Thus the values of ADF

Table I. Distribution of ADF and Actin in Chicken Tissues

	12–13-d Embryonic chick			
	Percentage of protein as		Molar ratio*	Adult chicken
Tissue	ADF	Actin	ADF/Actin	ADF as % soluble protein
Spinal cord	0.48	2.6	0.41	0.12
Brain	0.44	3.2	0.31	0.22
Intestine	0.37	2.4	0.34	0.33
Aorta	0.37	3.1	0.27	0.71
Gizzard	0.28	3.4	0.18	0.24
Kidney	0.23	0.8	0.64	0.40
Lung	0.20	1.1	0.41	0.11
Skeletal muscle	0.18	1.8	0.22	<0.01
Heart	0.09	1.2	0.17	<0.02
Liver	0.08	0.5	0.36	0.20
Serum	ND	ND	ND	0.00

ND, not determined.
* Molar ratios calculated using molecular weights for ADF of 19,000 and actin of 42,500.

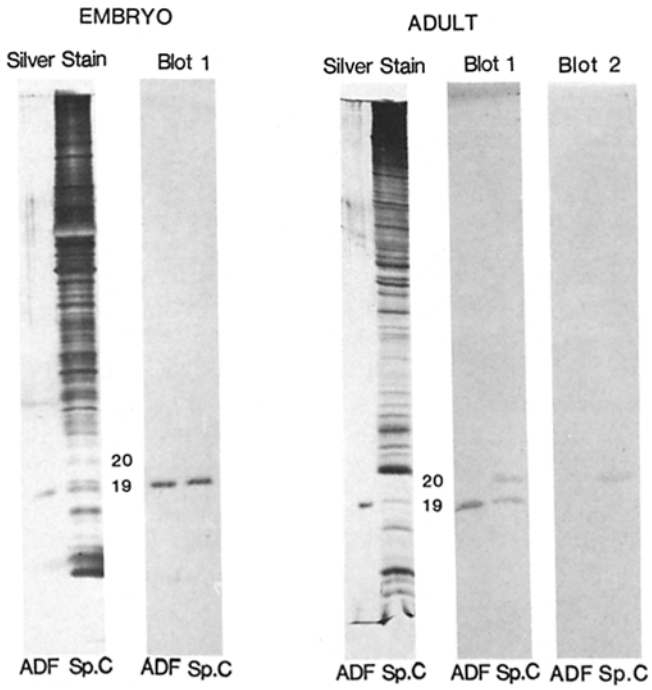

Figure 3. Appearance of higher molecular mass nonspecific immunoreactive band in chicken spinal cord during development. Silver-stained gels contained 10 μg of protein from spinal cord and 40 ng ADF. Gels for immunoblot contained 4 μg of spinal cord protein and 10 ng ADF. Immunoblot *1* was incubated with antiserum to ADF, whereas immunoblot *2* was incubated with preimmune serum at the same dilution.

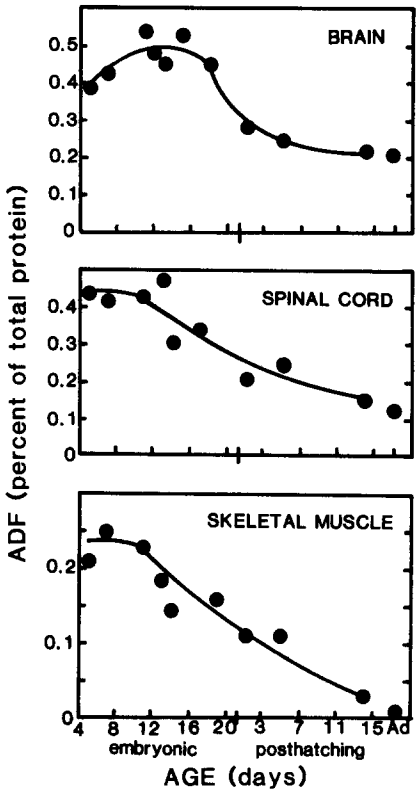

Figure 4. Developmental changes in ADF levels in chick brain, spinal cord, and muscle as measured by densitometry of immunoblots. Each immunoblot contained a series of ADF samples (3–30 ng) for use as a standard curve, and aliquots of tissue extracts containing from 3 to 20 μg total protein.

reported in Table I for the adult chicken are given as a percentage of the soluble protein. For some of these tissues (i.e., aorta) the amount of protein that was extracted was only a small fraction of the total and therefore the reported levels of ADF are deceptively high. However, three general conclusions can be made from the results shown in Table I: (*a*) No ADF is present in serum; (*b*) ADF virtually disappears in heart and skeletal muscle between the embryonic and adult chicken; (*c*) ADF levels remain high in smooth muscle and central nervous system tissues throughout development.

To determine if the immunoreactive form of ADF found in gizzard is similar biochemically to the brain ADF, two-dimensional immunoblots of the gizzard proteins were run. Two 19-kD species of ADF were observed on these blots, similar to those in Fig. 2. The two proteins had identical molecular masses and NEPHGE positions to the components of purified brain ADF, which was added to some of the gel samples as an internal marker (data not shown).

Developmental Changes in ADF

Immunoblots of adult tissue have one other interesting feature. A higher molecular mass band (20 kD) appears in brain, gizzard, and spinal cord but not in the other tissues examined. This 20-kD band becomes a major staining species in the spinal cord by 14 d posthatching (Fig. 3), but its appearance is not dependent on the presence of either primary or secondary antibody. Thus, this band probably possesses endogenous peroxidase activity.

The amount of ADF in brain and skeletal muscle was measured by densitometry of immunoblots prepared from chick tissues taken at different stages of embryonic and posthatching development (Fig. 4). ADF reaches a maximum level of 0.4–0.5% of the total protein in brain and spinal cord at ~10–12 d of embryonic development, a stage by which synaptogenesis is nearing completion and about the time when myelination beings (37). Posthatching, this level rapidly declines to the adult value of ~0.1–0.2%. Skeletal muscle from the thigh of the chick was used to study the disappearance of ADF during development. In the early embryonic stages of development (<9 d) it was difficult to obtain a piece of tissue that was known to be composed exclusively of muscle so the values for the younger ages are somewhat question-

Table II. ADF Levels in Specific Cell Types

Cell type	ADF as % total protein*
BHK/C21 (Hamster)	0.46
CHO (Hamster)	0.15
PC 12 (Rat)	0.22
HT 1080 (Human)	0.08
Neurons‡	0.58
Platelets§	0.28
Acanthamoeba	<0.01

* Estimated by comparison to chick brain ADF. The degree of immuno-cross-reactivity between ADF of different species is not known.
‡ Neurons from dissociated embryonic chick sympathetic ganglia cultured for 48 h in the presence of nerve growth factor.
§ Platelets from chicken blood.

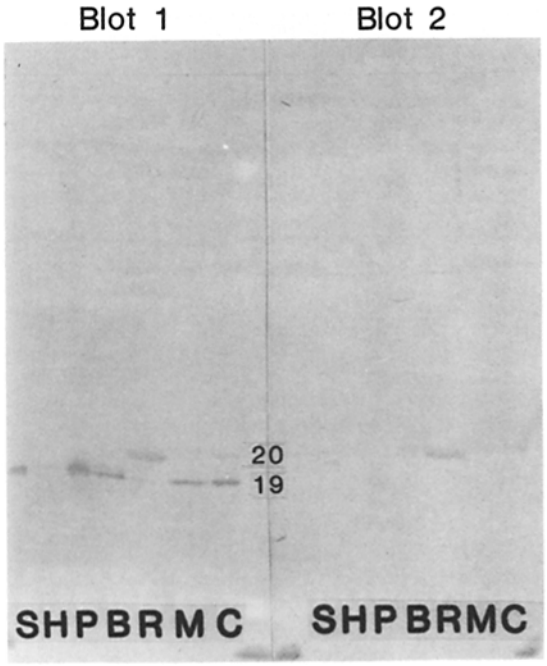

Figure 5. Immunoblots of extracts from nerve tissue and cell lines from several species of vertebrates demonstrating the distribution of protein with immunoreactivity to the ADF antiserum. Symbols for the different species and the amounts of protein loaded are: *S*, ADF (12 ng); *H*, HT 1080 (human fibrosarcoma) cells (6.6 μg); *P*, PC 12 (rat pheochromocytoma) cells (21 μg); *B*, BHK (baby hamster kidney) cells (11 μg); *R*, rabbit brain (24 μg); *M*, mouse brain (18 μg); *C*, chicken brain (7 μg). Immunoblot *1* was incubated with antiserum to ADF, whereas immunoblot *2* was incubated with the preimmune serum at the same dilution. Note the nonspecific staining of a 20-kD band in extracts from rabbit brain.

able. However, it is quite clear that the ADF levels start declining significantly by 14 d of embryonic development and ADF has almost disappeared by 14 d posthatching. These results were confirmed by immunofluorescence studies on frozen sections of fixed muscle described below. Therefore, the decrease observed in ADF levels in muscle during development is not due to a conversion of ADF to a nonextractable form.

Species Distribution of ADF

Cultured vertebrate cells (chick sympathetic neurons, BHK cells, CHO cells, rat pheochromocytoma [PC 12] cells, and human HT1080 cells) contained a specific immunoreactive protein as did platelets purified from chicken blood (Table II, Fig. 5), but none was found in the protozoan, *Acanthamoeba castellanii*. Immunoreactive forms of ADF were found in brain extracts of chicken, mouse, and rabbit, but not in nerve tissue from *Drosophila*, myxicola, or snail (Fig. 5). The major specific immunoreactive form of ADF was 19 kD, though a minor species of slightly higher molecular mass was present in some extracts. Although proteins with ADF activity have been isolated from both bovine (2) and porcine (29) brain, only very weak immunoreactivity was found in brain extracts from these species and from elk. Therefore, either very little ADF occurs in the adult brain of these species, or the ADF from these species is not highly reactive

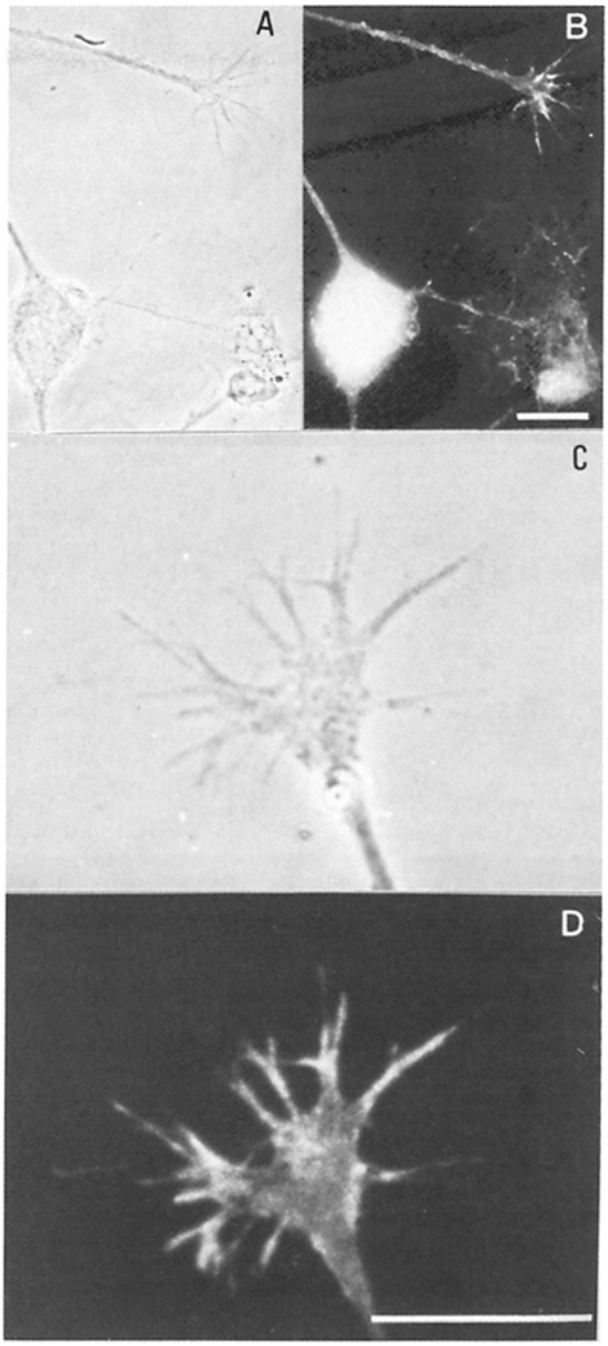

Figure 6. Indirect immunofluorescence localization of ADF in cultured cells from dissociated dorsal root ganglia of 9-d embryonic chicks. Phase-contrast photomicrographs (*A*, *C*). Fluorescence photomicrographs taken with rhodamine filters (*B*, *D*). Cells were incubated with either preimmune serum or antiserum to ADF, followed by biotin-labeled donkey anti-rabbit IgG and then Texas red–streptavidin. No staining occurred with preimmune serum (not shown). Bars, 10 μm.

to the antiserum. Immunoblots of extracts prepared from adult brain of many mammalian species and from chicken also contained a 20-kD band, a nonspecific staining protein that appeared in the absence of ADF antiserum (Fig. 5). This protein is absent in embryonic chick tissue.

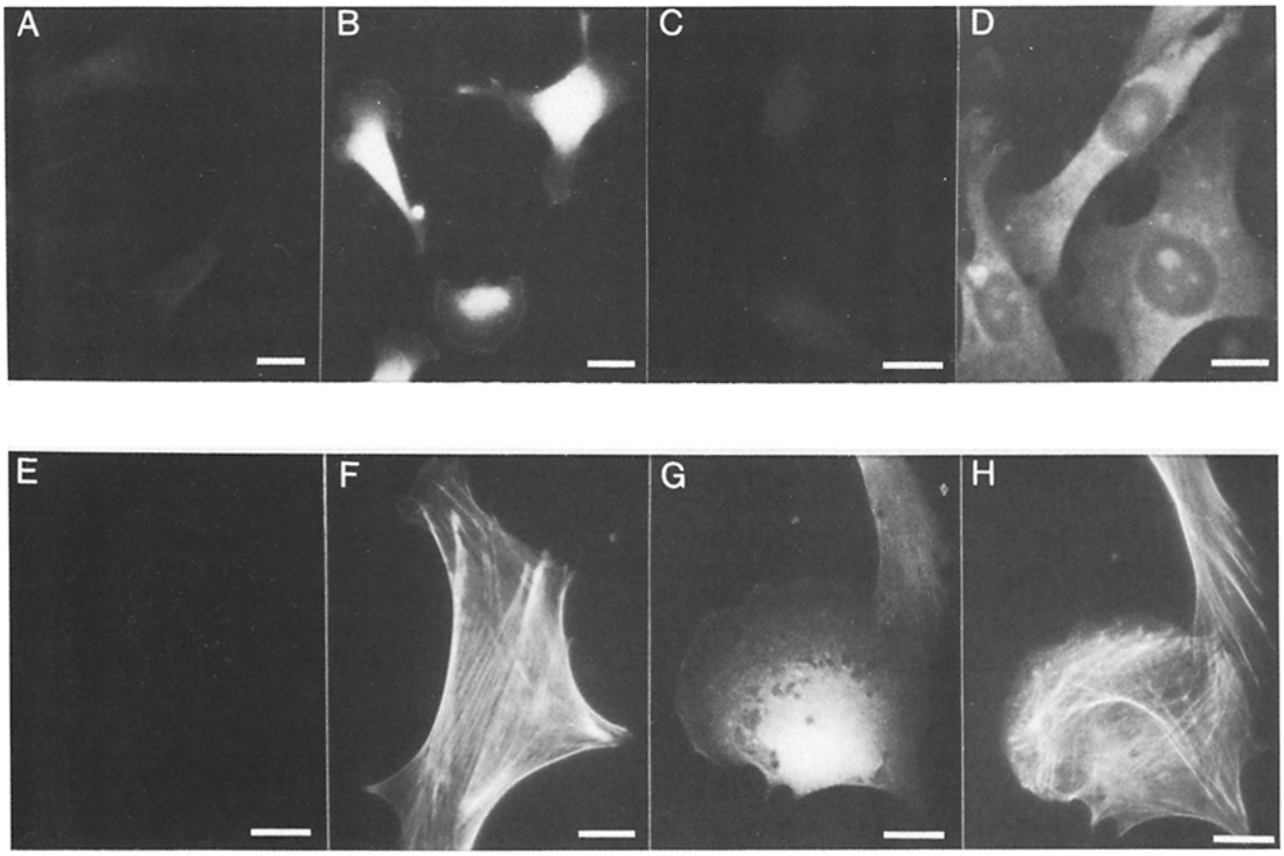

Figure 7. Distribution of ADF and actin filaments in cultured cells. *Top row*, chick skin fibroblasts (*A*, *B*) and BHK cells (*C*, *D*) were treated with a 1:100 dilution of preimmune serum (*a*, *c*) or anti-ADF antiserum (*B*, *D*). Cells were then treated with biotin-labeled donkey anti-rabbit IgG followed by Texas red-streptavidin, and were photographed with rhodamine filters. *Bottom row*, chick skin fibroblasts treated with preimmune serum (*e*, *f*) or ADF antiserum (*g*, *h*). Cells were then treated with fluorescein-labeled goat anti-rabbit IgG followed by rhodamine-phalloidin to visualize the actin filaments. Cells in *E* and *G* were photographed using the fluorescein filters while the same cells were rephotographed in *F* and *H*, respectively, with the rhodamine filter. The distribution of ADF in cells shown in *G* does not correspond to the distribution of actin filaments in the same cell shown in *H*. Bars, 10 μm.

Immunofluorescent Localization of ADF

Cultured cells from dissociated dorsal root ganglia prepared from 10-d chick embryos were fixed in various ways as described in Materials and Methods. Extraction with Triton X-100 before fixation resulted in the loss of most ADF immunoreactivity as observed by indirect immunofluorescence. Simultaneous fixation and extraction, or fixation followed by extraction resulted in well preserved cell morphology and retention of the ADF immunoreactivity (Fig. 6). ADF is present in growth cones, especially in the microspikes, and also along the neurite shaft. No immunoreactivity was observed in control cultures treated with an equal dilution of preimmune serum together with the fluoroconjugate (Texas red-streptavidin) except that neuronal cell bodies showed some background staining.

Non-neuronal cells in the dorsal root ganglia cell cultures also showed ADF immunofluorescence (Fig. 6). ADF localization in non-neuronal cells was studied in more detail in primary cultures of chick skin fibroblasts and in cultured BHK cells. Fibroblasts showed a general diffuse fluorescence with ADF antibody with slightly higher fluorescence at the leading edge of ruffling membrane (Fig. 7, *A* and *B*). ADF immunofluorescence visualized with fluorescein-conjugated goat anti-rabbit IgG did not correspond to actin immunofluorescence visualized in the same cell with rhodamine-phalloidin (Fig. 7, *G* and *H*). The lack of ADF staining in the nucleus was more readily observed in epithelial derived cell line, BHK-21/C13 (Fig. 7, *C* and *D*), though a bright staining of the nucleolus was observed in these cells.

Staining of tissue sections for ADF immunoreactivity confirmed the immunoblotting results which show a wide tissue distribution of ADF (Fig. 8). A variety of embryonic cell types contain ADF immunoreactivity. This is particularly well demonstrated in the sections of 10-d embryonic chick intestine (Fig. 8, *A–D*) where the different cell populations are clearly discernable in the phase-contrast photographs. Staining of cells in the developing mucosal layer, submucosal layer, and the circular layer of the muscularis externa are evident. Muscle from 10-d embryonic chick shows considerable ADF immunoreactivity which appears to be excluded from the central regions of the myofibers where sarcomere development is most pronounced (Fig. 8, *E* and *F*). Muscle from 17-d embryonic chick has considerably less ADF immunoreactivity (data not shown), confirming the immunoblotting results described above.

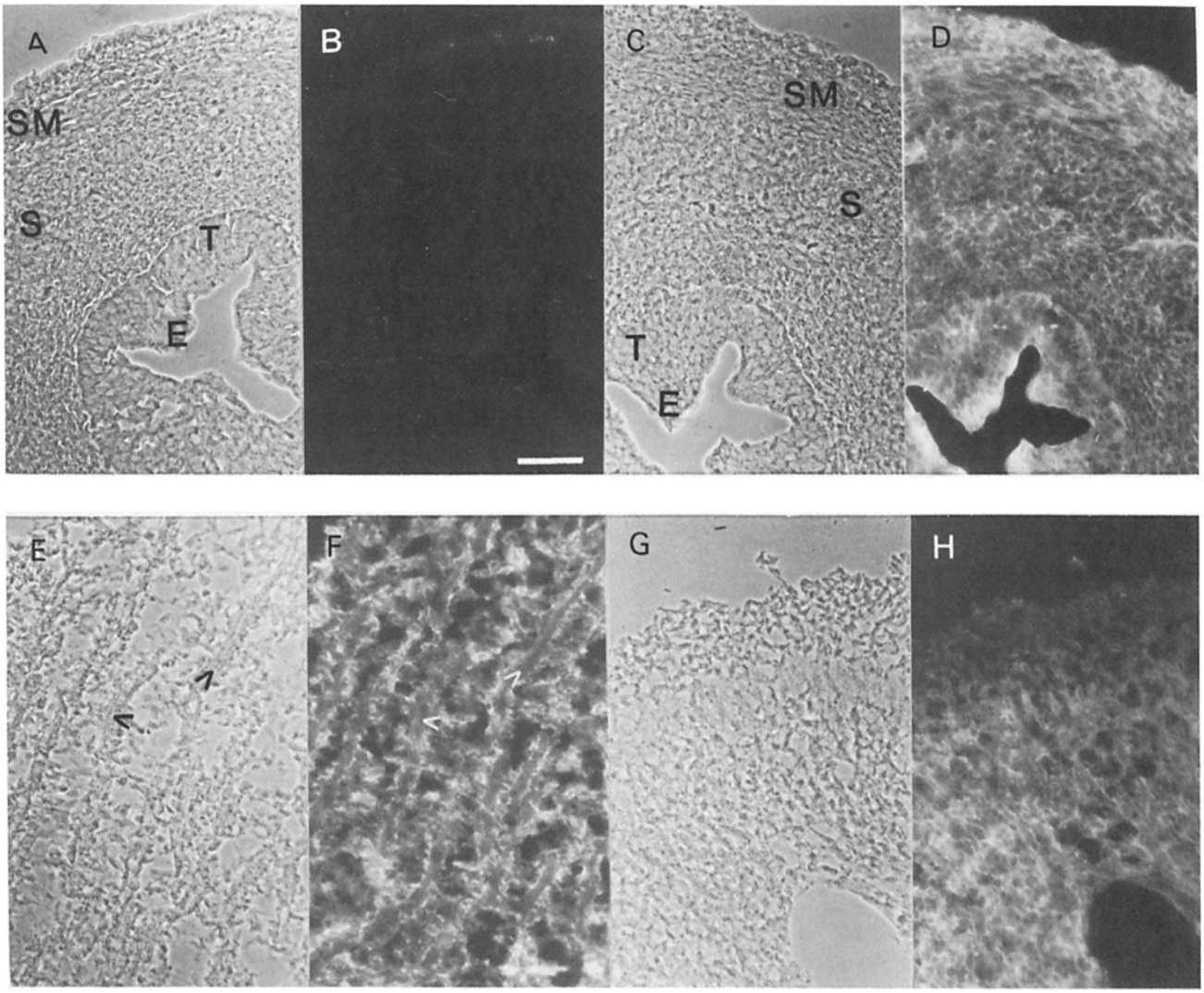

Figure 8. Localization of ADF in sections of embryonic chick tissues by indirect immunofluorescence. Sections (10-μm thick) were prepared from paraformaldehyde-fixed intestine (*A–D*) and muscle (*E, F*) from 10-d embryonic chick and cerebellum (*G, H*) from 17-d embryonic chick. Sections were treated with 1:100 dilution of preimmune serum (*A, B*) or anti-ADF antiserum (*C–H*) followed by Texas red–labeled donkey anti–rabbit IgG. Phase-contrast photomicrographs (*A, C, E, G*). Fluorescence photomicrographs (*B, D, F, H*) were taken using Texas red filters. Labeling on *A* and *C*: *E*, epithelial cells of mucosal layer; *T*, Tunica propria; *S*, submucosal layer; *SM*, smooth muscle cells of the muscularis externa. Arrows in *E* and *F* point to central region of developing myofiber in which ADF immunoreactivity is very slight. Bar, 50 μm.

Discussion

The term "actin depolymerizing factors" was originally applied to proteins from both serum and brain which rapidly depolymerized filamentous actin in vitro (1, 42). Serum ADF (also called brevin)—a secreted form of the 90-kD, Ca^{2+}-activated actin severing and capping protein, gelsolin—is now called serum gelsolin (45). Chick brain ADF is a 19-kD protein that depolymerizes F-actin by both severing actin filaments and sequestering actin monomers in a Ca^{2+}-independent manner (1, 15; Giuliano, K. A., F. A. Khatib, S. M. Hayden, E. W. Daoud, M. E. Adams, D. A. Amorese, B. W. Bernstein, and J. R. Bamburg, manuscript submitted for publication). Proteins of similar activity have been identified in starfish oocytes (23), mammalian brain (2, 29) and kidney (30), and *Acanthamoeba* (11). Since these proteins differ somewhat from each other in molecular mass and amino acid composition their relationship to each other has not been clear. In this paper we have demonstrated that a specific antibody raised against the chick brain ADF cross-reacts with proteins of identical molecular mass in a variety of cell types and in different vertebrate organisms. Based on the degree of cross-reactivity of this antibody to proteins from avians and mammals, one can conclude that some of the major antigenic determinants on the ADF molecule have been retained during evolution. ADF is an important actin assembly regulatory protein in vertebrates. Further studies are needed to elucidate the relationship of the vertebrate ADFs to the proteins of invertebrate cells that have both similar sizes and biochemical activity on actin but do not cross-react with the polyclonal antiserum to chick brain ADF.

Several higher molecular mass proteins are known to sever actin filaments and to bind monomeric actin (39, 42). Since, in addition, some of these proteins are potential substrates for proteases in vivo (38, 44), it was important to demon-

strate that ADF does not arise from the proteolysis of a higher molecular mass species. Despite careful tests in which we solubilized freshly dissected tissue samples by heating in detergent solution and then performed immunoblots from low percentage acrylamide gels, and thus obtained transfer of proteins of very high molecular mass, no higher molecular mass species were found that cross-reacted with the antiserum to ADF.

The disappearance of ADF from skeletal muscle and heart during development is notable in that these two tissues have much more of their actin in stable filaments than do other tissues. Since actin disassembly and reorganization do not occur on a large scale in these tissues after maturation, ADF may be unimportant in these tissues. That ADF immunoreactivity disappears during the period of development when muscle-specific genes are being activated (16) implies that the ADF gene is also developmentally regulated and that it is shut down together with non-skeletal muscle contractile proteins, such as the α-cardiac and β-cytoplasmic isoforms of actin.

The extractability of the ADF from unfixed cells with nonionic detergents and its diffuse localization in both cultured cells and frozen tissue sections are consistent with its abilities to form a soluble complex with monomeric actin. However we did observe a higher concentration of ADF along the leading edge of ruffled membranes in cultured fibroblasts (see Fig. 7) and in the microspikes of neuronal growth cones in culture (see Fig. 6). These locations are of interest because they may be sites of actin polymerization and depolymerization associated with cell locomotion (6, 41). It is relevant to note here that some ADF appears to be associated with the plasma membrane in fractions obtained from cultured BHK cells (Koffer, A., A. J. Edgar, and J. R. Bamburg, manuscript submitted for publication). It is possible, therefore, that the ADF–actin complex may serve as a reservoir of monomeric actin for filament assembly in response to appropriate membrane transducing signals in a manner analogous to that proposed for profilactin (22, 26). However, we wish to emphasize that ADF is not the same protein as profilin. The two proteins differ in molecular mass, immunoreactivity, and in their action on actin (28, 29). The ability of ADF to sequester actin molecules, even those in an already polymerized state, taken together with its abundance and widespread distribution, suggest that ADF, along with profilin, is a major factor in the regulation of actin filaments in many vertebrate cells.

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