Human Actin Depolymerizing Factor Mediates a pH-Sensitive Destruction of Actin Filaments

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ABSTRACT: ADF (actin depolymerizing factor) is an M_r 19 000 actin-binding protein present in many vertebrate tissues and particularly abundant in neuronal cells. We have cloned human ADF and here show it to be identical in sequence to porcine destrin. Human ADF expressed in *Escherichia coli* behaves like native ADF from porcine brain. It binds to G-actin at pH 8 with a 1:1 stoichiometry and $K_d \sim 0.2$ μM, thereby sequestering monomers and preventing polymerization. It does not cosediment with F-actin at this pH, but severs actin filaments in a calcium-insensitive manner. The severing activity is only about 0.1% efficient. By contrast, at pH values below 7, ADF binds to actin filaments in a highly cooperative manner and at a 1:1 ratio to filament subunits. When the pH is raised to 8.0, the decorated filaments are rapidly severed and depolymerized.

Actin depolymerizing factor (ADF)[1] was first isolated from chick embryo brains as an M_r 19 000 protein that promoted the disassembly of actin filaments (Bamburg *et al*, 1980). It was found to be widespread in adult mammalian and avian tissues including neuronal tissue, smooth muscle, kidney, heart, lung, and intestine, but the expression level was much higher in embryonic tissues (Bamburg & Bray, 1987; Abe *et al.*, 1989). Up to 0.4% of the total cell protein in tissue extracts from embryonic brain is ADF, and even higher concentrations have been reported in cultured neurones. Its role during development is unknown, but it is localized in the microspikes of growth cones of dorsal root ganglia (Bamburg & Bray, 1987). A role for ADF in conjunction with severing proteins like gelsolin has been suggested during the degeneration/regeneration of actin filaments at sites of neurosecretion (Bamburg & Bernstein, 1991).

ADF-like proteins have been isolated from both higher and lower eukaryotes. These include depactin from starfish oocytes (Mabuchi, 1983), porcine destrin (Nishida *et al.*, 1984a), and cofilin (Nishida *et al.*, 1984b), actophorin from *Acanthamoeba castellani*, (Cooper *et al.*, 1986), and yeast cofilin (Moon *et al.*, 1993; Iida *et al.*, 1993). The sequences of chick brain ADF (Adams *et al.*, 1990), porcine brain destrin (Moriyama *et al.*, 1990), porcine brain cofilin (Matsuzaki et al., 1988), and both ADF and cofilin from chick skeletal muscle (Abe *et al.*, 1990) show strong conservation throughout their length: even the yeast protein has 41% identity with vertebrate cofilins (Moon *et al.*, 1993). There is weaker homology with depactin (Takagi et al., 1988) and actophorin (Quirk *et al.*, 1993). A cofilin/ADF-like sequence has also been identified in the *unc-60* locus in *Caenorhabditis elegans* (Dr. D. L. Ballie, personal communication).

It has recently been demonstrated that disruption of the cofilin gene inhibited cell division in yeast, showing that cofilin is an essential component of the cell's cortical cytoskeleton (Moon *et al.*, 1993). Homozygous *unc-60* mutants in *C. elegans* result in abnormal thin filament organization and frequently in paralysis of the animals, again indicating the importance of cofilin/ADF-like proteins for normal muscle development (Waterston *et al.*, 1980).

Both ADF (Giuliano *et al.*, 1988) and cofilin (Nishida *et al.*, 1984b; Abe *et al.*, 1989) form 1:1 complexes with G-actin. Both proteins depolymerize F-actin at alkaline pH. Severing by ADF is also evident from the enhanced rate of actin polymerization in its presence (Bamburg *et al.*, 1991). The major difference reported between ADF and cofilin relates to their F-actin binding properties. Between pH 6.3 and 7.5, porcine cofilin cosedimented with F-actin, but at pH values between 7.5 and 8.3, it severed actin filaments but showed no stable binding (Yonezawa *et al.*, 1985). Switching from a higher to a lower pH gave increased polymerization and filament-bound cofilin: switching in the reverse direction induced depolymerization and monomer binding. These activities were confirmed for bacterially expressed cofilin, whereas ADF (destrin) showed no filament binding at the lower pH values (Moriyama *et al.*, 1990a). This F-actin-binding activity of cofilin may account for its intranuclear localization with cytoplasmic actin rods in response to heat shock (Nishida *et al.*, 1987), a process that is accompanied by the dephosphorylation of the cofilin (Ohta *et al.*, 1989).

In this paper, we show that human ADF is identical in sequence to porcine destrin: thus, ADF and destrin are the same protein. We further demonstrate that human ADF, expressed in *Escherichia coli*, shows a pH-dependent interaction with F-actin similar to that reported for cofilin. At pH values below 7.0, it binds to filaments, while at pH values above 7.5 binding occurs preferentially to monomers. We suggest that the intracellular activities of ADF may be regulated by small changes in pH, within established physiological ranges (Bright *et al.*, 1987). In addition, we quantitate the severing activity of ADF and show that it has low severing activity by comparison with members of the gelsolin family. Nevertheless, when actin filaments decorated

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[1] Abbreviations: ADF, actin depolymerizing factor; SDS–PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; DTT, dithiothreitol; E_M, molar extinction coefficient; PI-actin, actin labeled on Cys_{374} with *N*-pyrenyliodoacetamide; ε-ATP, 1,N^6-etheno-ATP; RhPh, tetramethylrhodamine phalloidin.

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with ADF are switched to a higher pH, there is rapid destruction of the filaments to monomeric form.

MATERIALS AND METHODS

Unless otherwise stated, all DNA manipulations were carried out as described in Maniatis *et al.*, (1982), and all restriction endonucleases and DNA modifying enzymes were purchased from New England Biolabs (Beverly, MA).

Isolation and Sequencing of Human ADF. A cDNA library prepared from 21-week-old human fetal brain mRNA in λgt11 (Clontech Laboratories Inc., Palo Alto, CA) was screened with a chick embryonic ADF partial cDNA [clone CB-2; Adams et al., 1990; kindly donated by Prof. J. R. Bamburg (University of Colorado, Fort Collins, CO)]. Primary screening of approximately 10^6 independent plaques yielded 1 positive clone, H1. The insert was fully sequenced on both strands by random "shotgun" cloning and sequencing as described by Bankier *et al.* (1987), and the data were assembled using the programs "SAP" and "DBUTIL" developed by Dr. R. Staden (L.M.B., Cambridge, UK).

Construction of the Expression Vector pMW172-ADF. The polymerase chain reaction was used according to the procedure of Clackson *et al.* (1991) to generate an *Nde*I restriction site at the 5′ end of the ADF coding sequence in order to express the gene in the vector pMW172. The DNA fragment generated was double-strand-sequenced, subcloned into the expression vector pMW172 as an *Nde*I–*Eco*RI fragment, and transformed into *E. coli* strain BL21(DE3) for expression (Way *et al.*, 1990).

Expression and Purification of ADF. Expression of ADF was carried out using the method of Way *et al.* (1992a). Cell extract supernatant was dialyzed overnight against ADF buffer (10 mM Tris-HCl, pH 8.0, 5 mM DTT, 0.2 mM EGTA, 1 mM sodium azide, and 0.1 mM phenylmethane sulphonyl fluoride), clarified, and filtered through a 0.2-μm filter (Millipore Continental Water Systems, Bedford, MA). The ADF was purified in the flow-through on DEAE-cellulose equilibrated in ADF buffer, concentrated using spin centrifugation to $A_{280} > 1$ (Centricon 30 spin concentrators; Amicon Corp., Davers, MA), and then gel-filtered on Sephacryl S200 in the same buffer. The concentration of ADF was determined from A_{280} values using a calculated extinction coefficient based on the tyrosine and tryptophan content, E_M = 11 210 (i.e., A_{280} = 1.00 is equivalent to 89.2 μM ADF). This value was confirmed using the Lowry assay (Weeds *et al.*, 1986). N-Terminal sequencing was performed to confirm the identity of the expressed protein.

Antisera to ADF were prepared as described by Bamburg & Bray (1987), and the antibody titer was determined using a "Vectastain ABC" immunoassay kit (Vector Laboratories, Burlingame, CA). The specificity of this antisera was tested using the expressed ADF, recombinant chick ADF, and cofilin prepared from baby hamster kidney cells (Bamburg *et al.*, 1991).

Preparation of Proteins. Destrin was purified together with cofilin from pig brain as described by Nishida *et al.* (1985). Actin and PI-actin (actin labeled on Cys_{374} with *N*-pyrenyliodoacetamide) were prepared as described by Weeds *et al.* (1986). Gelsolin was prepared as described in Way *et al.* (1989). Smooth muscle myosin II was prepared from glycerinated minced chicken gizzard muscle as described by Kendrick-Jones *et al.* (1983).

Assays for G-Actin Binding. (1) Gel Filtration. All manipulations were carried out in G-buffer (2 mM Tris-HCl, pH 8.0, 0.2 mM ATP, 0.2 mM DTT, 1 mM sodium azide, and 0.2 mM $CaCl_2$). ADF (0.75 mL, 182 μM) was mixed with G-actin (0.75 mL, 132 μM) and applied to a Sephacryl S-200 column. Fractions were analyzed by SDS–PAGE. Similar methods were used to analyze ADF binding to F-actin using Sephacryl S-400 and buffers containing 50 mM KCl, 1 mM $MgCl_2$, and 1 mM EGTA.

(2)Estimation of Dissociation Constant by Nucleotide Exchange. Unbound nucleotide was removed from gel-filtered G-actin (in G-buffer containing 10 mM Tris-HCl, pH 8.0) by the addition of Dowex 1X8 (BDH Biochemicals Ltd., Poole, Dorset, U.K.; a 20% volume of a 50% slurry equilibrated in the same buffer without ATP was added to the actin). Samples were incubated for 1 min at 21 °C, and the resin was removed by low-speed centrifugation. Dowex 1X8 treatment was repeated, and actin was labeled by incubation with 200 μM ε-ATP (Molecular Probes Inc., Eugene, OR) for 1 h at 21 °C. Unbound ε-ATP was removed by repeating the Dowex 1X8 treatment, and 20 μM ε-ATP was added back to prevent denaturation of the actin; 5 μM ε-ATP labeled G-actin was mixed with 0.5–10 μM ADF or porcine destrin in a 400-μL volume, and ATP (5 μL) was added to 1.25 mM to displace the ε-ATP from the actin [ε-ATP binds more weakly to actin than does ATP (Neidl & Engel, 1979)]. The exponential rate of ε-ATP displacement, measured by fluorescence (360-nm excitation, 410-nm emission), decreases when ADF complexes the actin. Thus, K_d, the dissociation constant for the complex, can be calculated, on the basis of the model of Bryan (1988) for actin-binding domains of gelsolin:

If k_{obs} is the rate constant for ε-ATP release, then assuming k_{+1} and $k_{+2} = 0$, $k_{obs} = \{k_{-1} + k_{-2}([ADF_T] - [complex])/K_d\}/\{1 + ([ADF_T] - [complex])/K_d\}$, where $[ADF_T]$ is the total ADF concentration, $[\epsilon\text{-ATP•actin}]_T$ the total concentration of ε-ATP•actin, $[complex] = \{z - (z^2 - 4[\epsilon\text{-ATP•actin}]_T[ADF]_T)^{0.5}\}/2$, and $z = [\epsilon\text{-ATP•actin}]_T + [ADF]_T + K_d$. This equation was fitted by nonlinear least-squares analysis using Kaleidagraph [Synergy Software (PCS Inc.), Reading, Pa] to obtain values of K_d.

(3) Effect of ADF on the Apparent Critical Concentration of Actin. The apparent critical concentration was measured at pH 8.0 as described by Cooper *et al.* (1984). Samples of 0–5 μM actin (containing 20% PI-actin) were polymerized overnight in the presence or absence of 1–2 μM ADF or cofilin. The steady-state fluorescence was measured at excitation and emission wavelengths of 366 and 384 nm, respectively.

Assays for Interaction with F-Actin. (1) Video Microscopy. Severing of F-actin labeled with tetramethylrhodamine phalloidin (RhPh; Molecular Probes) was observed in a flow cell (Kron *et al.*, 1991) using a method modified from Maciver *et al.* (1991). Smooth muscle myosin II (100 μL, diluted to 46.2 nM in "anti-bleach" buffer containing 25 mM Tris-HCl, pH 8.0, 25 mM KCl, 4 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 0.3% glucose, 0.1 mg/mL glucose oxidase, 0.02 mg/mL catalase, and 4 mg/mL bovine serum albumin) was infused into the flow cell, binding to the nitrocellulose-coated face. Unbound myosin was removed using "anti-bleach" buffer. F-Actin (100 μL of 230 nM protein containing 115 nM RhPh) was introduced and allowed to bind to the immobilized myosin. The flow cell was rinsed with 100μlof "anti-bleach"buffer and severing initiated by the infusion of ADF(50μL at 226 μM in"anti-bleach" buffer). Control infusions were carried

out with "anti-bleach" buffer instead of ADF to test the stability of the filaments. Samples were viewed by epifluorescence microscopy under oil immersion at an excitation wavelength of 546 nm with a Nikon Optiphot microscope using a Hamamatsu low-light camera, 63× magnification, as described by Kron *et al.* (1991).

(2) Viscometry. Capillary flow viscometry was performed as described by Way *et al.* (1989) using 17 μM F-actin in 1.0 mM calcium or 0.2 mM EGTA in the presence of ADF.

(3) Light Scattering Measurements. Light scattering was measured at an angle of 90° and 520-nm wavelength in a Perkin Elmer LS50B luminescence spectrophotometer.

(4) Severing Assay. Quantitation of the severing activity of ADF was determined from fluorescence assays in a Perkin Elmer LS50B luminescence spectrophotometer in two different ways.

(A) The kinetics of F-actin depolymerization were determined as described by Way *et al.* (1989), both in F-buffer conditions but also in modified G-buffer (10 mM Tris-HCl, pH 8.0, 0.2 mM ATP, 1 mM sodium azide, and either 0.2 mM $CaCl_2$ or 0.1 mM $MgCl_2$ + 0.2 mM EGTA) to ensure that depolymerization proceeded to completion; 6 μM F-actin (containing 20% PI-actin and polymerized ±60 nM gelsolin) was diluted to 200 nM, and the initial rate of depolymerization was measured from the rate of fluorescence decrease. ADF (0–320 nM) was added after approximately 60 s, and the rate constant for depolymerization was determined by nonlinear least-squares fitting of the exponential decrease in fluorescence (Way *et al.*, 1992a). Since the rate constant $k_{obs} = N/A^{\circ}(k_{-b} + k_{-p})$, where N = the number concentration of filaments, A° = 200 nM actin, and $k_{-b} + k_{-p}$ are the dissociation rate constants at two ends of the filaments [values taken from Pollard 1986)], the value of N can be related to the ADF concentration used.

(B) ADF was mixed with 10 μM F-actin for varying times, and the mixture was then added as nuclei at 1.25 μM F-actin to 5 μM G-actin (gel-filtered and containing 15% PI-actin) in F-buffer (G-buffer plus 1 mM $MgCl_2$ and 100 mM NaCl). Care was taken to minimize physical shearing of the filaments by using cut tips on the Gilson pipets and mixing the ADF with actin by gently sucking the mixture up and down the pipet 3 times only. The initial linear rate of fluorescence increase was converted to an on-rate for actin monomers by dividing by the total fluorescence enhancement for polymerization of 5 μM actin; this gives a rate = $(k_{+b} + k_{+p})NA^{\circ}$, where A° = 5 μM G-actin, N is the filament number concentration, and k_{+b} and k_{+p} are association rate constants.

It is a requirement of the depolymerization assay that the level of severing must be relatively low so that the severed filaments do not disassemble to any significant degree during the mixing time in the cuvette. The extent of depolymerization in the mixing time is indicated by the instantaneous drop in fluorescence of PI-actin. This requirement together with the need to use an actin concentration below the critical concentration limits the ADF concentration that can be used. F-Actin and ADF are mixed at much higher concentrations in the nucleation assay, which is an advantage if the affinity of ADF for F-actin is low. A further advantage of the nucleation assay is that this assay does not require that severing occurs within the mixing time [in the case of gelsolin, it occurs in <10 s (Way *et al.*, 1989)].

To investigate the reannealing of filaments, 10 μM F-actin was sonicated for 1–2 s at half-power in a Kontes microultrasonic cell disrupter, and 1.25 μM F-actin samples was added to nucleation assays at various times after sonication as in (B) above.

Sedimentation Assays. Binding of ADF to F-actin was analyzed by sedimentation methods as described by Pope and Weeds (1986). All manipulations were performed in 0.2 mM ATP, 0.2 mM DTT, 1 mM NaN_3, 0.2 mM $CaCl_2$, 3 mM $MgCl_2$, 100 mM NaCl, and 50 mM buffer (either Tris-HCl for pH values 9.0, 8.3, 8.0, and 7.5; imidazolehydrochloride at pH 7.0 and 6.5; or sodium succinate at pH 6.0). ADF was mixed with 20 μM F-actin at various molar ratios. A limited number of experiments were also carried out with porcine destrin. In all cases, 100-μL samples were incubated for 20 min at room temperature and pelleted at 135000*g* in a Beckman Airfuge. Supernatant fractions and pellets were analyzed by SDS–PAGE as described previously (Way *et al.*, 1992b).

RESULTS

Isolation and Sequencing of Human ADF cDNA. Screening the λgt11 embryonic brain cDNA library produced a single full-length clone (H1). The complete 1452 base pair nucleotide sequence was determined on both DNA strands, at a redundancy of approximately 5.5 (Figure 1). H1 contains an ATG initiator codon at nucleotides 75–77, followed by a single open reading frame encoding 165 amino acids ending in a stop codon at residues 568–570. The remaining 882 nucleotides of 3′-untranslated sequence contain a putative signal for polyadenylation (AATAAA, nucleotides 1407–1411).

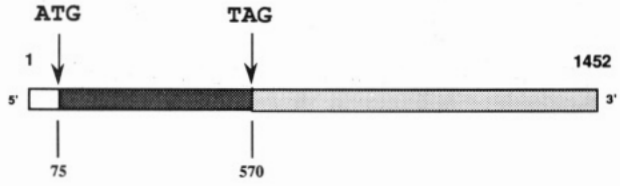
Human ADF is Identical to Porcine Destrin. The predicted sequence of the open reading frame of H1 is 100% identical to porcine destrin (Moriyama *et al.*, 1990a). Thus, ADF and destrin are the same protein. Human ADF shows 95% identity to chick ADF (Adams *et al.*, 1990; Abe *et al.*, 1990) and 70% identity to cofilins from porcine and mouse brain, chicken skeletal muscle, and human placenta (Matsuzaki *et al.*, 1988; Moriyama *et al.*, 1990b; Abe *et al.*, 1990; Ogawa *et al.*, 1991). There is almost complete conservation of the sequence motifs reported by Matsuzaki *et al.* (1988) and Adams *et al.* (1990) (Figure 1). Homology with actophorin from *Acanthamoeba castellanii* is much weaker (28%) (Maciver, unpublished results).

Expression of ADF. Figure 2 shows SDS–PAGE analysis of the purification of ADF. No additional purification was achieved using Matrix dye resins (Giuliano *et al.*, 1988). Yields of 10 mg of pure protein/L of bacterial culture were routinely obtained. The N-terminal amino acid sequence of the expressed protein (A-S-G-V-Q-V-A-D) confirmed that it was indeed ADF, but showed that the initiator methionine had been lost.

Antisera to the expressed ADF prepared in rabbits cross-reacted well with recombinant chick ADF but not with cofilin from baby hamster kidney cells. However, antisera prepared against chick ADF showed only weak cross-reactivity to the expressed human ADF (not shown). This difference in immune reactivity is surprising in view of the fact that there are only eight amino acid substitutions between the human and chick ADF sequences.

Preparation of Porcine Destrin. Destrin was prepared from pig brain as a control to compare with expressed ADF; it was separated from cofilin on hydroxylapatite as described by Nishida *et al.* (1985). SDS–PAGE showed single bands in each case, but the yields of pure protein were very low: about 1 mg of cofilin and less than 0.2 mg of destrin from 600 g of porcine brain. This recovery limited the experiments for which comparisons with bacterially expressed proteins could be carried out.

A.



B.

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CGGGGAGGACGGTCTGCATACTCGCTGCCCGCCGGCTCCCTCCCCCGCGTCCCTGCGACC   60

              M  A  S  G  V  Q  V  A  D  E  V  C  R  I  F  Y   16
GCCGCGGCGAAGATGGCCTCAGGAGTGCAAGTAGCTGATGAAGTATGTCGCATTTTTTAT  120

 D  M  K  V  R  K  C  S  T  P  E  E  I  K  K  R  K  K  A  V   36
GACATGAAAGTTCGTAAATGCTCCACACCAGAAGAAATCAAGAAAAGAAAGAAGGCTGTC  180

 I  F  C  L  S  A  D  K  K  C  I  I  V  E  E  G  K  E  I  L   56
ATTTTTTGTCTCAGTGCAGACAAAAAGTGCATCATTGTAGAAGAAGGCAAAGAGATCTTG  240

 V  G  D  V  G  V  T  I  T  D  P  F  K  H  F  V  G  M  L  P   76
GTTGGAGATGTTGGTGTAACCATAACTGATCCTTTCAAGCATTTTGTGGGAATGCTTCCT  300

 E  K  D  C  R  Y  A  L  Y  D  A  S  F  E  T  K  E  S  R  K   96
GAAAAAGATTGTCGCTATGCTTTGTATGATGCAAGCTTTGAAACAAAAGAATCCAGAAAA  360

 E  E  L  M  F  F  L  W  A  P  E  L  A  P  L  K  S  K  M  I  116
GAAGAGTTGATGTTTTTTTTGTGGGCACCAGAACTAGCACCTCTGAAAAGTAAAATGATC  420

 Y  A  S  S  K  D  A  I  K  K  K  F  Q  G  I  K  H  E  C  Q  136
TATGCAAGCTCCAAGGATGCAATTAAAAAGAAATTTCAAGGCATAAAACATGAATGTCAA  480

 A  N  G  P  E  D  L  N  R  A  C  I  A  E  K  L  G  G  S  L  156
GCAAATGGACCAGAAGATCTCAATCGGGCTTGTATTGCTGAAAAGTTAGGTGGATCCTTA  540

 I  V  A  F  E  G  C  P  V  *                               165
ATTGTAGCCTTTGAAGGATGCCCTGTGTAGATTATTCAGTGCCACAAATTGAAAGCTTCC  600

ATGTTTAATGTTATCCTCTTGCTATATAAATAAAGCAAATATATTTAGGCCAGGGTCTCA  660
CTGAGGGGGAGCTGTCTTGTCATCTTTTAGAGTAAACTATTCTATAAACATATGCAAACA  720
GCCCTAAATAAATCTAAAGTCTAAAGTTTTATTGATGTGAAATTAAATTCTTATTGGCCA  780
AATGCCTGTTTTGATGAGTTGATTTATAAAGATTTTTGTTAAGCTCAGGATTTTAAATTA  840
CACAGTTCACAAACAGTAAAGGCCATGTGAAGAGAATTATTACATCTTTATTAACCTCAG  900
CATTTACTTTGTTTCTTTTGCTTAGGAAATTGCTCATAATCTGGTTATAATTTTGGTCCA  960
AATTCTTTATTCTTCCTTGAGCTAAGCAGAATAATGGAATATAATATGTCTTCATAATAT 1020
AACAACACTAATACACTAATAGTAAGATTAAGTTAGGCAGTCTTCTACCAAATGTGTAAT 1080
GGAGATTGCCTCAAAATTGTGTCCACATAATCCACGCTCATCTTGCAAAGCGCTATTTCA 1140
GGCACATCATTGGAATACAGGAAGTAGCCCTGCACCTGCCAGTGAGCTCGCCATTCACTG 1200
ATTGGAAGAGTGACCTGGCATCTTGGAAATCATTGTGTGTCTTCAGGAGAATGTGCAGTG 1260
TCTTGTAACAACTAATTAATAATGCAAATTAGGGCTACATTGTAATCTGCTTTGTTAATG 1320
AAAATGATAAAACAGAATATTGACAAGCTAGGACACCTGTGGTATCTTTAATTGTATCTC 1380
CTTCAGAAGTTTGCTTCTTATGGTATAATAAAGTATGGAAGAATAAAAAAAAAAAAAAAT 1440
CTAGAGTCGCCG                                                  1452
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FIGURE 1: (A) Schematic representation of human ADF clone H1. The coding region (495 nucleotides) is indicated by the dark shaded box, and the two arrows indicate the start and stop codons. The 5′-noncoding sequence is shown as an unshaded box and the 3′-noncoding region by a light shaded box. (B) Nucleotide sequence and predicted amino acid sequence of clone H1 (ADF). The nucleotides and amino acids are numbered on the right side of the sequence. The polyadenylation signal from residues 1407–1411 is shown in boldface type.

Interaction of ADF with G-Actin. When ADF was mixed in slight excess with G-actin, the complex eluted from Sephacryl S-200 at an apparent molecular weight of 64 000, and gel densitometry of the 10 peak fractions gave a mean actin:ADF molar ratio = 0.95 ± 0.11, confirming the presence of a 1:1 complex.

Assays with fluorescently labeled actin previously used to measure complex formation between actin and gelsolin (Way *et al.*, 1989) proved unsuitable for ADF–actin complexes, because there was no fluorescence enhancement with either PI-actin or NBD-actin. Complex formation reduced the rate of nucleotide exchange on the actin as previously shown for gelsolin (Bryan, 1988): exchange kinetics were analyzed using the model developed by Bryan. Figure 3 compares the effects of ADF and porcine destrin on the rate of ϵ-ATP displacement from G-actin. A K_d value of 0.14 + 0.1 μM for the G-actin–ADF complex was calculated by nonlinear least-squares fitting. Similar values were obtained for at least three different preparations. The K_d for porcine destrin is likely to be similar, although, as Figure 3 shows, there was insufficient protein to

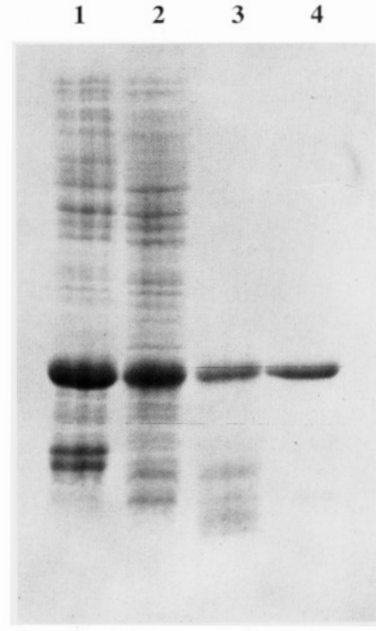

FIGURE 2: Expression and purification of recombinant human ADF. SDS–PAGE of (1) total cell lysate after overnight incubation, (2) soluble bacterial fraction, (3) protein not retained on DEAE-cellulose, and (4) protein eluted from the Sephacryl S200 gel filtration column at an estimated molecular weight of 18 000.

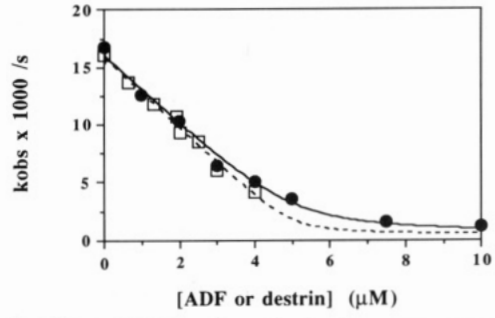

FIGURE 3: Effect of ADF and porcine destrin on the rate constant for the exchange of ϵ-ATP bound to 5 μM G-actin by 1.25 mM ATP. ADF (filled circles); destrin (open squares).

cover a complete range of concentrations. Experiments with cofilin suggested a slightly higher K_d of 0.5 μM.

A K_d value was also obtained from critical concentration measurements (Figure 4). Using two different ADF concentrations, a mean value of 0.3 μM was obtained. Porcine destrin gave a similar value of 0.5 μM. It should be noted that there is no evidence from these measurements that ADF has a high affinity for the barbed ends of filaments—low concentrations of ADF did not produce a disproportionately large increase in the apparent critical concentration of the actin consistent with barbed-end capping, as previously reported for the monomer-binding domain of gelsolin (Way *et al.*, 1992a,b). However, it should be emphasized that a half-maximal increase in monomer concentration requires capping of about 99% of the actin filaments (Wanger & Wegner 1985), which means that the capping protein must have a very high affinity for filament ends.

pH Dependence of ADF Binding to F-Actin. ADF cosediments with F-actin at pH 6.5 but not at pH 8.0 (Figure 5A). The binding curve is strongly sigmoidal, with a Hill coefficient, calculated by nonlinear least-squares analysis, of 3.6 (Figure 5B). This suggests strong cooperativity, confirmed by the biphasic Scatchard plot. The maximum level of binding is 1:1 with actin subunits.

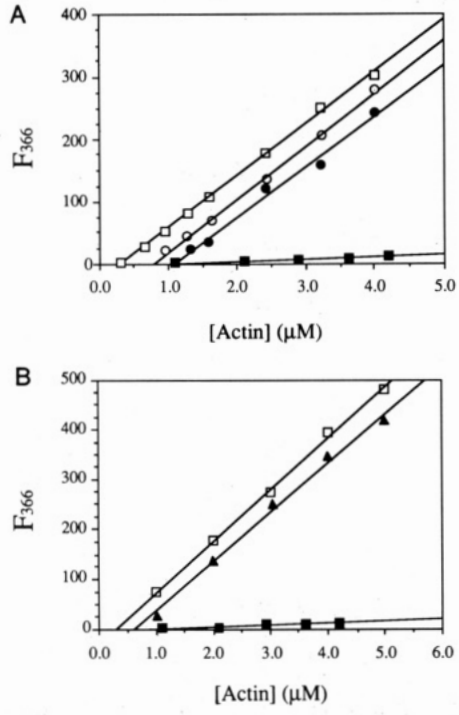

FIGURE 4: Effect of ADF, porcine destrin, and porcine cofilin on the steady-state concentration of polymerized actin measured by the fluorescence of pyrenyl actin. (A) Unpolymerized actin (filled squares); actin polymerized in the absence of binding protein (open squares); actin polymerized in the presence of 1 μM ADF (open circles) and 2 μM ADF (filled circles). (B) As in (A) except that solid triangles are for actin polymerized in the presence of 1.3 μM porcine destrin. The dissociation constants for the complexes were as follows: ADF = 0.3 μM; porcine destrin = 0.5 μM.

Binding experiments were carried out at a number of different pH values as shown in Figure 5C. The majority of the actin was present in the supernatant together with ADF at pH values between 7.5 and 9.0, but at pH values between 6.0 and 7.0, both actin and ADF were present predominantly in the pellet. It can be seen that there is a transitional phase at about pH 7.5 where a small drop in pH shifts the majority of ADF from the supernatant to the pellet. Although we were unable to carry out a full series of experiments with porcine destrin, the overall conclusions were the same: using a somewhat lower destrin:actin ratio of 1:4, about 60% of the actin appeared in the supernatant at pH 8.0 together with over 90% of the destrin, while at pH 6.5, over 98% of the actin was pelleted with 90% of the destrin (Hawkins, 1992).

Gel filtration of complexes of ADF with F-actin showed that both proteins were eluted in the void volume on Sephacryl S-400 at pH 6.5, but at pH 8.0, they coeluted in the position of complexes with G-actin.

Evidence for Actin Severing by ADF. Direct evidence for filament severing by ADF was obtained using video microscopy. When ADF was infused into a flow cell containing actin filaments partially labeled with rhodamine phalloidin and attached to the glass slide via smooth muscle myosin, there was an immediate but small decrease in the fluorescence intensity of the filaments. Within about 30 s, the filaments showed a fragmented appearance, and fluorescent debris was observed in the areas between the filaments as shown in Figure 6A. Control filaments showed uniform fluorescence throughout their length, and there was minimal photobleaching. Infusion of anti-bleach buffer alone had no effect on the filaments, and the areas between filaments were devoid of fluorescent debris (Figure 6B). Photobleaching was also

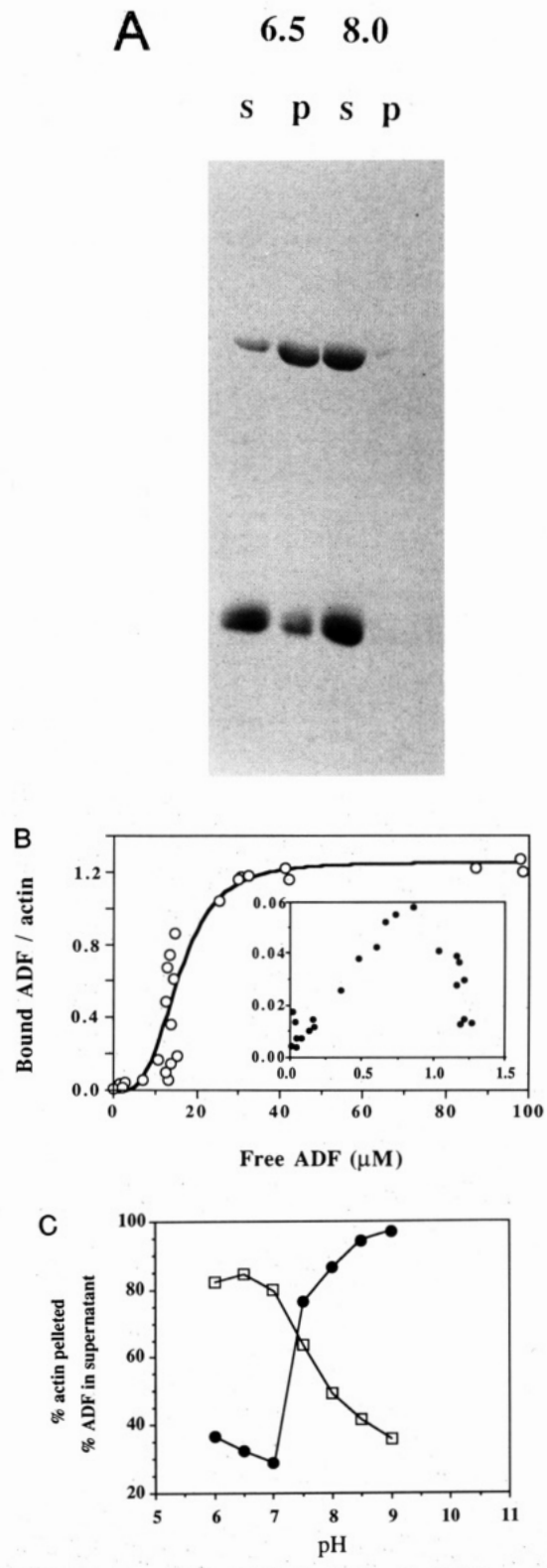

FIGURE 5: Cosedimentation of ADF with F-actin. (A) Supernatant and pellets obtained after centrifugation of 20 μM actin in the presence of a 4-fold molar excess of ADF. Densitometry of the pelleted material at pH 6.5 gave an ADF:actin molar ratio of 1.3. (B) Binding of ADF to F-actin at pH 6.5. Bound ADF has been expressed as a molar ratio to actin subunits. Nonlinear least-squares fitting shows maximum binding at 1.2 ADF per actin and a Hill constant of 3.69. 50% binding occurs at a free ADF concentration of 14 μM. The insert shows a Scatchard plot of the same data, with [bound ADF/actin] on the abscissa and {[bound ADF/actin]/[free ADF]} on the ordinate. (C) Relative percentages of sedimentable actin and ADF in the supernatant when 1:1 mixtures were centrifuged at different pH values in an airfuge. Percent of actin in the pellet (open squares) and percent of ADF in the supernatant (filled circles).

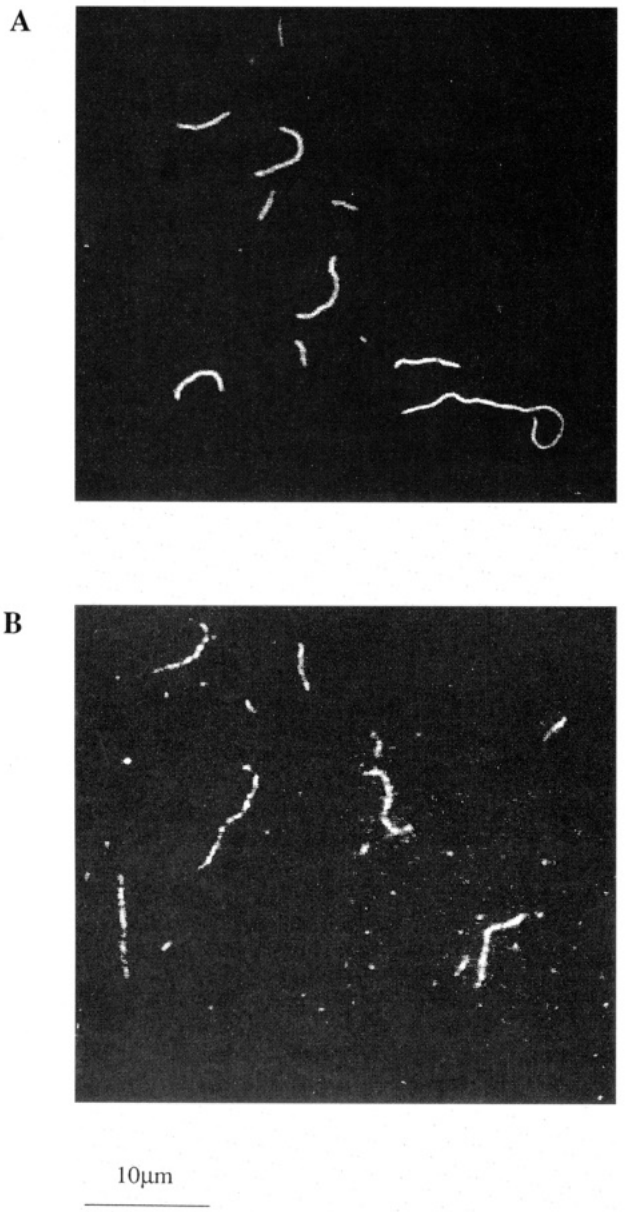

FIGURE 6: Video light micrograph of 50% rhodamine phalloidin labeled actin filaments before and after severing by ADF. (A) Actin filaments 30 s after infusion of anti-bleach buffer alone. (B) Actin filaments 30 s after the infusion of ADF. The same filaments could not be visualized before and after infusion of ADF as lateral movement of the microscope stage upon addition of ADF was unavoidable.

minimal in the control sample: thus, the instantaneous quenching of fluorescence on addition of ADF may be caused by its binding transiently to the sides of the filaments prior to severing them.

Indirect evidence of the severing by ADF was obtained by viscometry. Figure 7 shows two experiments at pH 8 and a single experiment at pH 6.5. The viscosity falls to a similar extent at substoichiometric ratios of ADF to actin under both conditions. No differences were oberved in the effects of ADF ± calcium. At saturating concentrations of ADF, there is a much larger drop in viscosity at pH 8 than at pH 6.5. For example, when 34 μM ADF was added to 17 μM F-actin at pH 6.5, the specific viscosity fell from 0.59 to 0.25. Addition of 6 μL of 2 M Tris-HCl, pH 8.5 (1% volume), to raise the pH to >8.0 resulted in a further fall in viscosity to <0.05 in less than 2 min, the time needed to make the measurement. (There was no change in viscosity when the same volume of pH 6.5 buffer was added as a control.)

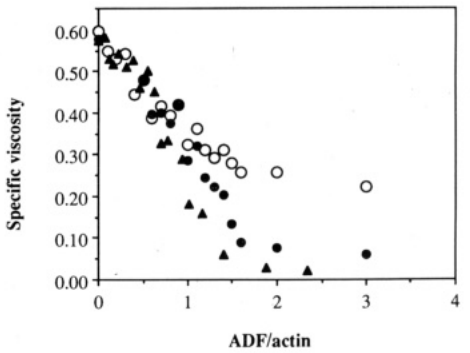

FIGURE 7: Effects of ADF on the specific viscosity of 16.8 μM F-actin in the presence of calcium at pH 8.0 (open circles and closed triangles, two sets of experiments) and at pH 6.5 (closed circles).

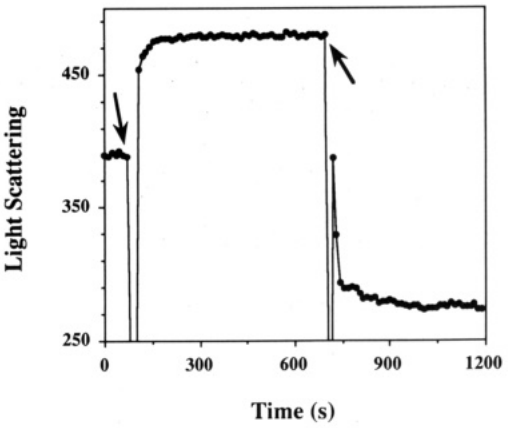

FIGURE 8: Effects of the addition of 20 μM ADF (first arrow) to 15 μM F-actin at pH 6.5 on the light scattering of actin. At the second arrow, the pH was increased to 8.0 by addition of 5% buffer volume. The final level of light scattering was identical to that of G-actin and ADF at the same concentration and pH.

Because of the time delay involved in viscometric measurements, light scattering was used to monitor the speed of disassembly of F-actin. Addition of ADF to filaments at pH 6.5 resulted in a rapid rise in light scattering, consistent with ADF binding (Figure 8). Raising the pH to 8.0 gave an almost instantaneous drop in light scattering to a value identical to that of a mixture of ADF and G-actin at the same concentrations.

Quantitation of Severing Activity. The effects of ADF on the rate constants of depolymerization of capped filaments (actin polymerized in the presence of 1% gelsolin) are shown in Figure 9A. The values of the slopes are 0.119 $(\mu M\ ADF)^{-1}$ s^{-1} in low salt and 0.0153 $(\mu M\ ADF)^{-1}$ s^{-1} in high salt. Since $k_{obs}/N = (k_{-b} + k_{-p})/A^o$, where N = filament number (related to the ADF concentration), A^o = 200 nM actin, and the k values are the rate constants for subunit dissociation at the two ends of the filaments (Way *et al.*, 1989), it is possible to calculate the frequency of severing from known rate constants or, assuming 100% severing, estimate the rate constants. Assuming that ADF is 100% efficient in severing activity, the values for $k_{-b} + k_{-p}$ are 0.0238/s and 0.00306/s, respectively. These compare with published values for the off-rate constants for ADP–actin subunits of 7.2/s and 0.27/s at the barbed and pointed ends, respectively, in 50 mM KCl, 1 mM $MgCl_2$, and 1 mM EGTA (Pollard, 1986); i.e., our calculated values suggest that severing is less than 0.1% efficient. Although the apparent severing activity under G-buffer conditions is 8 times higher, this ratio is similar to that of the initial rates of depolymerization of uncapped filaments measured under identical conditions.

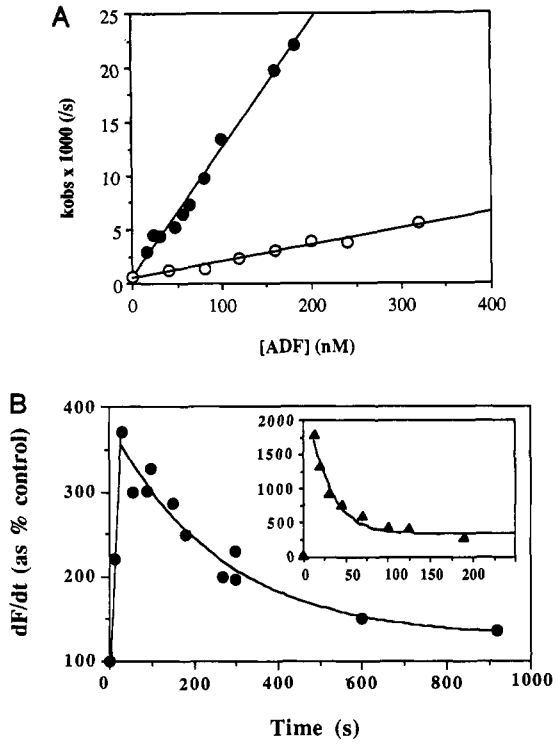

FIGURE 9: (A) Effect of ADF on the rate constants for F-actin depolymerization in the severing assay at pH 8.0 in G-buffer conditions (closed circles) and in F-buffer (open circles). (B) Effect of ADF on the nucleating activity of F-actin at pH 8. The nucleation rate was measured at different times after mixing 10 μM F-actin with 12 μM ADF; the F-actin or mixtures with ADF were added at 1.25 μM to 5 μM G-actin (containing 15% PI-actin), and the initial rate of fluorescence increase (dF/dt) was measured. Values are relative to controls in the absence of ADF. The decline in the observed rate is fitted to an exponential with rate constant 0.00382/s, corresponding to a half-time for reannealing of 180 s. The inset shows the reannealing of sonicated F-actin in a similar assay (axes as in panel B). The rate constant for this exponential is 0.0403/s, corresponding to a half-time for reannealing of 17 s.

These experiments suggest that the severing activity of ADF is extremely low. This is not unexpected because for this assay to work well, severing must occur instantaneously and the severing protein must have a high affinity for actin subunits, because the proteins are mixed at an actin concentration below the critical concentration and at ADF concentrations in the same range or lower. An alternative approach was to mix the ADF with F-actin at much higher concentrations and measure the increase in filament number in a nucleation assay. When ADF was mixed 1:1 with 10 μM F-actin, the nucleating activity varied with time after mixing. Maximum activity, about 4 times the control rate, occurred after 40–100 s, and the nucleation rate declined thereafter to control levels within about 20 min (Figure 9B). These results suggest that severing is not instantaneous. Using the association rate constants of Pollard (1986) for ATP–actin monomers under conditions closest to those here (5.7 and 0.8 $\mu M^{-1} \cdot s^{-1}$ in 1 mM $MgCl_2$ but at pH 7 and without NaCl), the filament concentration calculated from the elongation rate on addition of 1.25 μM F-actin to 5 μM G-actin was 0.20 nM in the absence of ADF and 0.8 nM in its presence. Thus, the maximum increase in filament concentration obtained by mixing 12 μM ADF with 10 μM F-actin suggests a severing efficiency of less than 0.1%, similar to that estimated in the depolymerization assays.

The time-dependent decline in activity suggests a slow reduction in the concentration of nuclei. Nonlinear least-squares fitting indicates a half-time of 180 s (Figure 9B). The most obvious cause for this is filament reannealing. The inset in Figure 9B shows the effects of reannealing on the nucleating activity of F-actin after sonication. Assuming that reannealing is occurring in both cases, the 17-s half-time shows that sonication produces much shorter filaments than does severing by ADF. Reannealing is expected to occur at much faster rates when the filament number is high and their lengths short. At higher concentrations of ADF, the maximum nucleating activity occurred at shorter times, consistent with this hypothesis.

DISCUSSION

We have shown here that human ADF and porcine destrin are the same protein. Although the 2 protein sequences are identical, there are 16 base changes within the coding region (Figure 1; Moriyama *et al.*, 1990a). Comparison between human and chick ADF shows 78 differences in the nucleotide sequence, which result in 8 amino acid substitutions. Virtually all of these are conservative: the one significant change (alanine at position 42 in the human protein for proline in chick ADF) may account for the differences observed in immunological cross-reactivity.

ADF was expressed in active form and high yield in *E. coli*. Although we were able to prepare only limited amounts of porcine destrin for comparison of activities between the native and bacterially expressed proteins, no differences were found in any of the assays used. We therefore conclude that the bacterially expressed protein functions in an identical manner to native ADF. Similar conclusions have been reported for porcine destrin expressed in *E. coli* (Moriyama *et al.*, 1990a).

The main conclusions of this work are (i) the severing activity of ADF, quantitated for the first time, appears to be very low compared to gelsolin. However, it should be noted that the ADF:actin ratios in chick embryonic tissues (~0.2–0.4; Bamburg & Bray, 1987) are an order of magnitude higher than the gelsolin:actin ratios in highly motile cells (Kwiatkowski, 1988). (ii) Under the conditions used here and in contrast to earlier reports (Nishida *et al.*, 1985; Moriyama *et al.*, 1990a), ADF binds to F-actin at neutral or slightly acidic pH values, showing that cofilin and ADF are not qualitatively different in these properties. (iii) Binding is strongly cooperative, and (iv) decorated filaments are rapidly disassembled when the pH is shifted from 6.5 to 8.0.

Human ADF Binds Stoichiometrically to Monomeric Actin. ADF forms a 1:1 complex with monomeric actin. The dissociation constant is about 0.2 μM both from ϵ-ATP exchange and from critical concentration measurements (Figures 3 and 4) and there was no evidence for significant levels of barbed end capping [see Way *et al.* (1992a) for details of arguments]. Comparisons between expressed ADF and porcine destrin showed similar binding affinities for the two proteins. Our results compare well with those obtained for chick ADF (Bamburg *et al.*, 1991; Hayden *et al.*, 1993) and with earlier experiments on porcine destrin and cofilin (Nishida, 1985; Nishida *et al.*, 1985).

Human ADF Severs Actin Filaments. Clear evidence is presented that ADF severs actin filaments. This is most persuasively seen by light microscopy (Figure 6). Similar observations have been made using the related proteins actophorin (Maciver *et al.*, 1991) and gelsolin (Bearer, 1991), but at much lower molar ratios to actin. It should be emphasized that the actin used in these experiments was only half-saturated with rhodamine phalloidin, because ADF, like cofilin, does not bind to F-actin saturated with phalloidin (Hayden *et al.*, 1993; Yonezawa *et al.*, 1988). These observations together with electron micrographs of filaments

treated with ADF (not shown) suggest that the severing frequency is very low. Unlike the situation with gelsolin where nanomolar concentrations produce substantial severing, micromolar concentrations of ADF are required to obtain significant severing activity. This low severing activity was confirmed in other assays. Concentrations of ADF in excess of the actin concentration were required to reduce the specific viscosity of F-actin to that of G-actin (Figure 7), whereas gelsolin had a similar effect at <1% of the actin concentration (Way *et al.*, 1989). Nishida *et al.* (1984a, 1985) reported similar effects on viscosity when destrin was added to F-actin and suggested that these results showed a 1:1 stoichiometry between the two proteins.

Quantitative analysis of severing by ADF was carried out in both depolymerization and nucleation assays. These assays are a measure of filament number concentration, since rate constants observed for elongation or disassembly depend only on the number concentration (N) of filaments involved and the relevant association or dissociation rate constants. Using published rate constants (Pollard, 1986), both methods showed an increase in the number of filaments that was less than 0.1% of the ADF added.

Do these experiments reflect true severing rates? Values for N calculated from depolymerization or polymerization rates (Figure 9) clearly depend on the reliability of the rate constants. Although our conditions are not identical to those of Pollard (1986) or others published elsewhere (Carlier, 1991), rate constants do not vary by more than a factor of 2–5 at different pH and ionic conditions. It seems unlikely, therefore, that the choice of rate constant will significantly affect our conclusions. It is further assumed that ADF does not cap the barbed ends of filaments, justifying the use of combined rate constants at both ends in our calculations. Evidence against extensive capping at the barbed end comes from critical concentration measurements in the presence of ADF (Figure 4). Further evidence may be adduced from the time-dependent decrease in elongation rate when ADF was mixed with F-actin in the nucleation experiments (Figure 9B). This decrease was qualitatively similar to that observed using sonicated F-actin and is highly suggestive of filament reannealing. Although the rates of reannealing obtained from these assays are much slower than those reported by Murphy *et al.* (1988), this is expected if the severing frequency is low.

The very low level of severing estimated from these experiments may seem surprising in view of the total disassembly of filaments observed in sedimentation assays at pH 8.0. However, in view of the time taken, it might be argued that the sedimentation experiments could be explained solely by dissociation of subunits from filament ends and their subsequent sequestration by ADF. Comparative experiments on the effects of ADF and the monomer-sequestering C-terminal domain of gelsolin, which has an order of magnitude higher affinity for monomers than ADF (Way *et al.*, 1989), show that ADF increases the concentration of actin in the supernatant instantaneously, whereas an equivalent concentration of the gelsolin domain requires several hours of incubation to depolymerize filaments (B. Pope, unpublished observations). Severing must therefore play an important part in the filament disassembly.

F-Actin Binding Is Regulated by pH. Sedimentation analysis shows that ADF binds to filaments at pH 6.5 but not at pH 8 and that binding is strongly cooperative (Figure 5). As there is no evidence for self-association of ADF on its own, on the basis of gel filtration of ADF at high concentrations, it appears that the cooperativity reflects the effects of binding to actin. Cooperative binding of a similar nature has been observed with the actin-binding domain of dystrophin (Way *et al.*, 1992c) but not with the related domain from α-actinin (Way *et al.*, 1992b). Further work will be needed to establish whether the actin structure is affected by ADF binding.

Figure 5C shows that there is a sharp transition of ADF from the filament-bound state to the soluble fraction, accompanied by a relatively large shift of actin from the sedimentable to the nonsedimentable form when the pH is increased from about 7 to 7.5. Nishida *et al.* (1984) reported similar properties for cofilin but claimed that destrin did not bind F-actin (Nishida *et al.*, 1985). Our results demonstrate that there are no qualitative differences between cofilin and ADF in this regard. We are unable to account for the differences between our experiments and those published elsewhere (Nishida *et al.*, 1985; Moriyama *et al.*, 1990a, 1992), although one possible explanation concerns the exact pH conditions used in both sets of experiments. Another difference is that the bacterially expressed porcine destrin is believed to contain a nine-residue N-terminal extension (Moriyama *et al.*, 1990a), whereas our ADF is complete with the exception of the initiating methionine residue.

We have used viscometry and light scattering to extend our studies on the interaction of ADF with F-actin. Figure 7 shows that there is little difference in the effects of ADF on the viscosity of F-actin at pH 8 and 6.5 for ADF:actin ratios of <1. One possible explanation is that low levels of severing occur at both pH values; this has been confirmed in nucleation and depolymerization experiments at the two pH values (data not shown). The major differences in viscosity occur at ADF:actin ratios >1.5, i.e., under saturating conditions. At pH 8, both viscometry and sedimentation show that the actin is in monomeric form while at pH 6.5 most of it remains filamentous. At the lower pH, filaments are fully decorated with ADF (Figure 5). Raising the pH to 8.0 results in a rapid fall in viscosity and light scattering; measurements show that this conversion to values consistent with G-actin is very rapid indeed (less than half a minute). This suggests that decorated filaments exposed to a rise in pH are instantaneously depolymerized. Further experiments are in progress to quantitate these effects.

Possible Cellular Regulation of ADF Funtion. On the basis of the data presented, we propose that at pH values above 7.5 ADF forms binary complexes with G-actin with a $K_d \sim 0.2$ μM, similar to the critical concentration of F-actin. Thus, it will act as an efficient monomer-sequestering protein in the presence of filaments. In addition, it severs filaments at low frequency, and binary complexes of ADF with actin subunits dissociate from the filaments. This accelerates the depolymerization process by increasing the concentration of filament ends. By contrast, at pH values below 7, ADF binds stoichiometrically to filament subunits in a cooperative manner, but not significantly to G-actin. Increasing the pH from 7.0 to 7.5 results in almost instantaneous depolymerization of the ADF-bound filaments. Further work is in progress to analyze these effects in greater detail *in vitro* and to explore the localization of ADF in cells under different conditions.

Since the activity of ADF is regulated by pH *in vitro*, it is important to establish whether pH changes occur within this range in cells. Alkalinization of cells is well established in response to growth factors acting through protein kinase C. For example, Bright *et al.* (1987) reported the intracellular pH of motile fibroblasts was 7.35 compared to 7.1 in quiescent cells. Although this change is small, the localized rise in pH might be significantly greater. On the basis of our model, a

consequence of alkalinization would be increased severing activity and actin filament solation. This would result in increased motility, as has previously been shown when levels of gelsolin expression are increased in fibroblasts (Cunningham et al., 1992).

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