Differential Regulation of Actin Depolymerizing Factor and Cofilin in Response to Alterations in the Actin Monomer Pool*

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Myoblasts, transfected with a human gene encoding a β-actin point mutation, down-regulate expression of actin depolymerizing factor (ADF) and its mRNA. Regulation is posttranscriptional. Expression of cofilin, a structurally similar protein, and profilin, CapG, and tropomodulin is not altered with increasing mutant β-actin expression. Myoblasts expressing either human γ-actin or the mutant β-actin down-regulate the endogenous mouse actin genes to keep a constant level of actin mRNA, whereas the γ-actin transfectants do not down-regulate ADF. Thus, ADF expression is regulated differently from actin expression.

The mutant β-actin binds to ADF with about the same affinity as normal actin; however, it does not assemble into normal actin filaments. The decrease in ADF expression correlates with an increase in the unassembled actin pool. When the actin monomer pool in untransfected myoblasts is increased 70% by treatment with latrunculin A, synthesis of ADF and actin are down-regulated compared with cofilin and 19 other proteins selected at random. Increasing the actin monomer pool also results in nearly complete phosphorylation of both ADF and cofilin. Thus, ADF and cofilin are coordinately regulated by posttranslational modification, but their expression is differentially regulated. Furthermore, expression of ADF is responsive to the utilization of actin by the cell.

Regulation of the synthesis and assembly of cytoskeletal components is critical to cell survival. Although it has been recognized for many years that cellular demand for cytoskeletal components can drive the synthesis of monomer (1, 2), the autoregulatory mechanisms have been determined in detail only for β-tubulin (3–5).

Actin is also subject to feedback regulation although the mechanism is not established (2). Overexpression of human genes encoding γ-actin or a β-actin single mutant (β(sm)-actin) (Gly-244 to Asp) in mouse C2 myoblast cells results in the down-regulation of endogenous mouse β- and γ-actin genes, thus maintaining a constant level of actin mRNA and protein (6). In contrast, overexpression of a human gene encoding a highly unstable mutant actin protein fails to elicit down-regulation of the endogenous mouse genes. This suggests that the feedback regulation is directed by the protein product of that introduced gene (6).

Feedback regulation of actin synthesis by an increased monomer pool may involve the control of mRNA stability. Synthesis of actin decreases in cells that have been treated with the actin depolymerizing drugs, latrunculin A (Lat A) and botulinum toxin C2 (7–9). The decline in rates of actin synthesis in Lat A and C2 toxin-treated cells can be largely accounted for by the observed decrease in mRNA stability within the cytoplasm (8, 9). On the other hand, de novo synthesis of actin mRNA increases in cells in which actin assembly has been promoted by incorporation of phalloidin (9), suggesting that two different autoregulatory control mechanisms, one nuclear and the other cytoplasmic, work to maintain actin homeostasis.

Actin is also capable of reprogramming the expression of microfilament-associated proteins. High level expression of different human actin genes in mouse C2 cells impacts on the expression and organization of different tropomyosin isoforms (10) and on the expression of vinculin and talin (11). Vinculin expression is also decreased by drugs that depolymerize actin, and vinculin down-regulation requires the presence of a nucleus (8). In addition, changes in actin and vinculin expression in response to drug treatment are independent of changes in cell shape (8). High level expression of tropomyosin Tm1 (12), vinculin (13, 14), α-actinin (15), and gelsolin (16), which all alter cell morphology, have no impact on the expression of other microfilament proteins. This suggests that the ability of actin to reprogram expression of microfilament proteins may be unique to actin.

The assembly of the actin cytoskeleton depends upon the production and activities of a large number of actin binding proteins (reviewed in Refs. 17–19). Among the most important of these proteins are those in the profilin, thymosin, and actin depolymerizing factor (ADF)/cofilin families that have been implicated in regulating actin assembly in a number of different systems (reviewed in Refs. 20 and 21). How responsive the cellular levels of these proteins are to actin utilization is unknown. The mutant human β(sm)-actin is known to form aberrant actin filaments (22), and thus, clones of cells expressing different amounts of this protein provide a good model system to determine which of the actin assembly regulatory proteins are responsive to its nonfilamentous accumulation. Clones of

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cells expressing equivalent amounts of the assembly competent, normal human β- and γ-actins provide useful controls.

In various C2 cell clones, expressing human β-actin and normal human β- and γ-actins, we examined the levels of three actin monomer binding proteins ADF, coflin, and profilin. In addition, we looked at the expression of CapG (23) and tropomodulin (24), two proteins present in myoblasts that can also alter actin assembly dynamics and filament lengths. The expression of only one of these proteins, ADF, is inversely proportional to the levels of the assembly mutant, β-actin. Further analysis of the same C2 cell clones showed an increase in G-actin concomitant with a decrease in ADF expression, suggesting that ADF may be a critical assembly regulatory protein sensitive to the utilization of actin by the cell. To explore this hypothesis further, untransfected C2 cells were treated with Lat A to depolymerize F-actin and increase the unassembled actin pool. As previously shown in Swiss 3T3 and HeLa cells (8), the Lat A treatment induced a down-regulation in actin synthesis. Results presented below demonstrate that the synthesis of ADF, but not coflin, decreases when the pool of unassembled actin increases. In addition, C2 cells respond to Lat A by nearly complete phosphorylation (inactivation) of both ADF and coflin. Thus, although the posttranslational regulation of ADF and coflin is coordinated, their synthesis is regulated independently. We propose that perturbations in the actin monomer pool regulate the expression of ADF, but not coflin, in C2 cells.

MATERIALS AND METHODS

Purification and Quantification of Proteins

The following proteins were generous gifts of the individuals listed: cofilin, profilin and CapG from Dr. Helen Yin, University of Texas Southwestern Medical School; tropomodulin from Dr. Mark Sussman, University of Southern California. ADF was isolated from chick embryo brain (25). Recombinant chick ADF (26) and recombinant coflin (27) were prepared in the laboratory as described previously. Skeletal muscle actin was purified from rabbit muscle actin powder (28), and brain actin was purified from 18-day embryonic chick brain (29). Protein concentrations were determined by the filter paper dye-binding method, using ovalbumin as a standard (30).

Cell Culture

The C2C12 murine myoblast cell line was maintained as a monolayer in 75-cm² tissue culture flasks (Corning Laboratories, Corning, NY) in growth medium containing Dulbecco's modified Eagle's medium (low glucose), 20% fetal bovine serum, and 0.5% chick embryo extract (all from Life Technologies, Inc.). For experiments, cells were cultured in 6-cm or 10-cm tissue culture dishes at less than 30% confluence. All actin-glucose), 20% fetal bovine serum, and 0.5% chick embryo extract (all growth medium containing Dulbecco's modified Eagle's medium (low

Cell Nuclear Run-on Transcription

C2 cells (2–5 × 10⁷) were washed with 4°C PBS, lysed into SDS extraction buffer (10 mM Tris, pH 7.5, 2% SDS, 10 mM NaF, 5 mM dithiothreitol (DTT), 2 mM EDTA) by scraping, and the extracts were heated in boiling water for 3 min. After cooling, the samples were sonicated briefly and the proteins precipitated (35). The proteins were dissolved in 2 × sample preparation buffer (1 × buffer contains 0.125 × Tris, pH 6.8, 0.5% SDS, 5% glycerol, 5% 2-mercaptoethanol, 0.0005% bromphenol blue) for SDS-PAGE.

SDS-PAGE and Immunoblotting—SDS-PAGE was performed by the method of Laemmli (36) on 15% total acrylamide (2.7% cross-linker) isocratic mini-slab gels. Proteins were transferred electrophoretically to polyvinylidene difluoride (Immobilon P, Millipore Corp., Bedford, MA) for 1 h at 0.3 A in the buffer of Towbin et al. (37), using a Genie Electroboblotter (Idea Scientific, Minneapolis, MN), blocked, washed, and immunostained as described previously (25). Alkaline phosphatase-conjugated secondary antibody (Sigma) was diluted in the wash buffer. Blots were developed with Lumiphos (Boehringer Mannheim) after a quick rinse in 100 mM Tris, pH 9.5, 100 mM NaCl, 50 mM MgCl₂, or with CDP-star (Tropix Inc., Bedford, MA) after a quick rinse in 50 mM Tris, pH 9.5, 100 mM NaCl, 1 mM MgCl₂ to get exposures with the linear range of the Hyperfilm ECL (Amersham Corp.). Following chemiluminescent detection, blots were immunostained with NBT/BCIP (Life Technologies, Inc.) according to manufacturer's directions. Exposures of the chemiluminescence images and the stained blots were analyzed with a Microscan 2000 image analysis system (Technology Resources Inc., Knoxville, TN). Internal standards of proteins were included on every immunoblot.

Antibodies

Mouse monoclonal antibody (C4) to actin was purchased from ICN Pharmaceuticals, Inc. (Costa Mesa, CA). γ-Actin-specific rabbit anti-serum was a gift from Dr. J. Chloe Bulinski, Columbia University, NY. Monoclonal antibody to the β-actin isoform was from Sigma. Rabbit polyclonal antibody to ADF was prepared by us (38). Mouse monoclonal antibody (MaBi22) to coflin was a gift from Drs. Hiroshi Abe and Takashi Ohi, Chiba University, Japan (39). Polyclonal rabbit antibodies to human profilin and CapG were a gift from Dr. Helen Yin, University of Texas Southwestern Graduate School, Dallas, TX. Affinity purified rabbit IgG to tropomodulin was a gift from Dr. Mark Sussman, University of Southern California, Los Angeles, CA.

Immunoprecipitation

Supernatants from the homogenates of C2 myocyte cultures were prepared by scraping the washed cells from a 6-cm culture dish in 300 μl of 10 mM HEPES, pH 7.4, 0.15 mM NaCl, 1 mM DTT, and 1% Triton X-100 (IP buffer), sonicating the cell suspension for 3 s, and centrifuging at 10,000 × g for 5 min at 4°C. The supernatant was then preincubated on a rotator with 240 μl of a 1:1 suspension of protein A-agarose in IP buffer at 4°C for 5 min. The sample was centrifuged 10 s at 10,000 × g and the supernatant divided into two tubes. Anti-ADF IgG (2 mg/ml; 120 μl) or preimmune serum was added to each tube, and the tubes were rotated at 4°C overnight. Protein A-agarose (60 μl of a 1:1 suspension) in IP buffer was added to each tube (rotated at 4°C) for 1 h. The resin was centrifuged, washed once with 300 μl of IP buffer, and the bound antibody complexes extracted with 30 μl of 1% SDS in a boiling water bath. After centrifugation, the supernatant was mixed with an equal volume of 4 × sample preparation buffer for SDS-PAGE and immunoblotting.

Affinity Chromatography

The actin isoforms binding to ADF were also examined by using ADF-Affi-Gel resin. All steps were carried out at 4°C and under nitrogen. Recombinant ADF (5.9 mg) was dialyzed against degassed 0.1 mM MOPS, pH 7.5, for 4 h and then added to 1 ml of Affi-Gel 10 resin (Bio-Rad) hydrated in degassed MOPS buffer. After mixing for 2 min, 0.1 ml of 1 M ethanolamine, pH 8, was added, and the sample was mixed for 1 h. DTT (1 mM; 3 μl) was then added (nitrogen atmosphere was not needed after this step), and the resin was placed in a column and washed with 25 ml of MOPS buffer containing 1 mM DTT. Supernatants of extracts from cultured C2 cells or C2 transfectants were prepared as described under immunoprecipitation except that the lysis buffer was 0.1 M Tris, pH 8.0, and 1 mM EDTA (100 μg/ml of tissue). Supernatant (100
μl was added to 60 μl of a 1:1 suspension of the ADF resin in 0.1 M MOPS, pH 7.5, 1 mM DTT, or to the same volume of Sepharose 4B resin as a control. The samples were incubated on a rotator for 5 min at 4 °C. After microcentrifuging for 10 s, the resin was washed once with 300 μl of 1 mM Tris, pH 9.0, 1 mM DTT. The bound proteins were extracted with 40 μl of SDS at boiling water bath. Samples were diluted with an equal volume of 4 × sample preparation buffer, and actin isoforms were identified by SDS-PAGE and immunoblotting.

RNA Blotting and Analysis

RNA was isolated from duplicate sets of four 10-cm plates of C2 cells and each of the transfected clones using the guanidinium thiocyanate method (34). RNA from the duplicate samples was separated by agarose-formaldehyde electrophoresis, transferred to Hybond-N+ membrane (Amerham Corp.), and hybridized with a 260-base pair fragment of the human ADF cDNA (338–599 HindIII fragment; Ref. 40) which had been labeled with 32P by the random primer method (41) or with an 18 S ribosomal RNA probe (used in excess), end-labeled with T4 polynucleotide kinase, for normalization of loading the gel (6). Hybridization of the ADF probe was carried out in 4 × SSC, 5 × Denhardt’s solution (42), 50 mM NaH2PO4, and 10% dextran sulfate at 65 °C overnight and for 55 °C in 4 × SSC, 5 × Denhardt’s solution (42), 50 mM NaH2PO4, and 10% dextran sulfate at 65 °C overnight. Filters were washed 4 × for 30 min at 50 °C in 0.1 × SSC, 0.1% SDS for the ADF probe, and 2 × for 30 min at room temperature and 3 × for 20 min at 55 °C in 4 × SSC, 0.1% SDS for the 18 S rRNA probe. Autoradiography was performed at −70 °C with either Kodak XAR-5 film or Amersham Hyperfilm-MP. RNA bands on the autoradiogram (substantiation of the single bands) were quantified using the Microscan 2000 image analysis system. The integrated density of each ADF mRNA band was normalized to the amount of 18 S RNA in that sample with the value from wild type C2 cells taken as 100%.

Quantification of G-actin in Cultured Cells

Cultured cells were washed free of medium with four washes of 4 °C PBS and lysed in 10 mM Tris, pH 7.5, 2 mM MgCl2, 0.5 mM DTT, 2 mM EGTA, 1% Triton X-100, and 7.5% glycerol. Material was scraped to the edge of the plate and transferred with a wide bore pipette to a microcentrifuge tube for G-actin quantification or to an airfuge tube for preparation of supernatant and cytoskeletal fractions. The amount of G-actin in each lysate was determined by the DNase I inhibition assay (43, 44) using DNase I calibrated with purified skeletal muscle G-actin. Supernatant and cytoskeletal (pellet) fractions were prepared from the cell extracts by centrifugation of the lysates at 170,000 × g for 20 min. Actin in each fraction was determined by immunoblot analysis.

Preparation of Triton-soluble and Cytoskeletal Fractions

Lat A (a generous gift from Ilan Spector, SUNY, Stony Brook, NY) was added to C2 cell cultures to 5 μM for 0, 6, 12, and 18 h at 37 °C. The cells were washed four times with PBS (2 ml) and the remaining levels of ADF were extracted at room temperature with 50 mM MES, pH 6.5, 1 mM EGTA, 50 mM KCl, 1 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 0.5% Triton X-100, and 0.5% protease inhibition mixture (45) prepared at −70 °C with either Kodak XAR-5 film or Amersham Hyperfilm-MP. RNA bands on the autoradiogram (substantiation of the single bands) were quantified using the Microscan 2000 image analysis system. The integrated density of each ADF mRNA band was normalized to the amount of 18 S RNA in that sample with the value from wild type C2 cells taken as 100%.

Measurement of Rates of Protein Synthesis

C2 cells on 6-cm dishes were treated with Lat A (5 μM) for 0, 6, 12, and 18 h at 37 °C. Thirty minutes prior to the end of the Lat A treatment, the cells were washed twice with prewarmed D-mannose-, cysteine-, and serum-free Dulbecco’s modified Eagle’s medium (labeling medium). The cells were then incubated with 200 μCi/ml [35S]methionine/cysteine (Promix; Amersham Corp.) in labeling medium containing Lat A (except for control dishes) for 30 min at 37 °C. The labeling solution was aspirated and the cells washed twice with 2 ml of PBS at room temperature. The cells were lysed in 150 μl dish SDS extraction buffer and the extracts immediately heated to boiling for 3 min. After cooling, the samples were sonicated briefly, the proteins precipitated (35), and then dissolved in 9.5 μl urea, 5% 2-mercaptoethanol, and 2% Nonidet P-40 or Igepal (Sigma).

Proteins were separated by two-dimensional PAGE using nonequi-
expression are mouse actin gene expression (mRNA) as a percent of the total actin levels of mouse and human actin gene expression (6). The levels of human manner, we examined these cell lines transfected with the ADF or if other actin-binding proteins behave in an identical line (Fig. 1). Western blots shows that there is a strong inverse relationship between both the relative amounts of ADF mRNA and ADF levels (Fig. 1). Quantitative analysis of both Northern and Western blots of total RNA, extracted from these same clones were previously characterized for levels of mouse and human actin gene expression (6). The levels of mouse actin gene expression (mRNA) as a percent of the total actin expression are C2 (100%), LK56 (100%), 411 (83%), 425 (97%), 33B1 (31%), 33A5 (75%), 522 (35%), and 547 (97%). The only clone showing a significant difference in ADF expression from controls is 522, the highest expressing clone of the βsm-actin.

Northern blots of total RNA, extracted from these same βsm-actin expressing cell lines, show a decline in ADF mRNA levels (Fig. 1B). Quantitative analysis of both Northern and Western blots shows that there is a strong inverse relationship between both the relative amounts of ADF mRNA and ADF protein and the amount of βsm-actin expressed in each clonal line (Fig. 1C).

To determine if this type of regulatory response is specific to ADF or if other actin-binding proteins behave in an identical manner, we examined these cell lines transfected with the human βsm-actin gene for expression of cofilin, profilin, CapG, and tropomodulin by Western blotting. Cofilin expression remained constant with increasing βsm-actin expression (Fig. 2) demonstrating that the feedback regulatory signals differentially impact on the expression of these two structurally and functionally similar actin assembly regulatory proteins. Expression of profilin, CapG, and tropomodulin also remained constant with increasing βsm-actin expression (Fig. 2). The mutant βsm-actin is therefore highly specific in its effect on actin binding proteins.

Possible Mechanisms for the Observed Changes in ADF Expression—To determine if there is a relationship between the mechanisms responsible for changes in ADF expression that occur in the βsm-actin expressing clones and those that alter endogenous actin gene expression and/or cell morphology, we examined clonal cell lines of C2 cells transfected with the normal human γ-actin gene. Previous studies have revealed a remarkable similarity between the impact of βsm-actin and γ-actin gene transfections in the C2 cells. Both genes lead to down-regulation of the endogenous β- and γ-actin genes and a similar decrease in cell surface area (6, 31). In addition, both genes produce down-regulation of the tropomyosin isoforms Tm2,3 (10), and the focal adhesion proteins vinculin and talin (11). No significant difference in the level of ADF expression (as a percentage of total protein) was found in either the high or low expressing clones resulting from transfection of the human γ-actin gene (Fig. 3). This demonstrates that neither morphology nor endogenous actin gene expression was directly coupled to the ADF down-regulatory response. The down-regulation of ADF was not due to intrinsic activity of an exogenous β-actin promoter. Clones expressing mRNA encoding a normal human β-actin at levels comparable with those of the highest βsm clone (6) did not impact on ADF expression (Fig. 3).

To examine the level at which ADF expression was down-regulated, we compared the transcriptional activity of ADF, actin, and 18 S RNA genes in wild type C2 cells with that in the highest βsm-actin-expressing clone (clone 522) by nuclear run-on assays (Fig. 4). Normalizing the transcriptional activity to 18 S RNA, the ratio of expression (522 cells/wild type) for ADF is 1.6, and for actin it is 0.56. Neither of these ratios is significantly different from 1, but the ADF ratio is significantly different from 0.2, the value expected if ADF expression is totally regulated at the level of transcription, since the ADF mRNA level in the 522 cells is about 20% of that in wild type C2 cells. These results suggest that ADF down-regulation is controlled posttranscriptionally.

ADF Recognizes and Binds to Mutant βsm-Actin Protein—It seemed possible that ADF expression could be down-regulated by the decreased levels of normal actin expressed by the cell. Indeed, the level of normal β-actin closely parallels the level of
ADF in these cells. ADF might not recognize the $\beta_{\text{sm}}$-actin pool, and thus, the level of ADF could be regulated by the size of the pool of normal actin. To determine if ADF would recognize and bind to the $\beta_{\text{sm}}$-actin, we used two approaches. First we lysed the C2 cell line expressing the highest level of $\beta_{\text{sm}}$-actin (clone 522) into immunoprecipitation buffer and immunoprecipitated the ADF and associated proteins with a rabbit ADF antisera. The immunoprecipitates were washed, solubilized in SDS, and the actin isoforms associated with the ADF were identified by immunoblotting using a monoclonal antibody that recognizes all of the actin isoforms (Fig. 5A). For the second approach, we used an ADF affinity resin to bind actin in lysates of the cell line expressing the highest level of $\beta_{\text{sm}}$-actin. The bound actin was extracted with SDS, and isoforms were identified by immunoblotting (Fig. 5B). Both methods demonstrate that ADF binds the $\beta_{\text{sm}}$-actin in the same ratio to total actin as occurs in the whole cell lysate.

**ADF Activity and Expression Are Perturbed by Increasing the Actin Monomer Pool**—Since the $\beta_{\text{sm}}$-actin mutation does not affect ADF binding, it seemed likely that the levels of ADF might depend upon the utilization of actin by the cell and be regulated by signals dependent upon the monomer or filamentous actin pools. To assess the distribution of actin isoforms between the soluble and cytoskeletal pool, 170,000 × g supernatant and pellet fractions were prepared from extracts of C2 cells and three of the $\beta_{\text{sm}}$-actin expressing cell lines used above. The distribution of $\beta$-actin, $\beta_{\text{sm}}$-actin, and $\gamma$-actin in the supernatant and pellet fractions were determined from Western blots, using a polyclonal antibody specific for the $\gamma$-actin isoform and a monoclonal antibody specific for the $\beta$-actin and the aberrantly migrating $\beta_{\text{sm}}$-actin isoforms (Fig. 1A). The results of this analysis (Table I) show that in cells expressing more of the $\beta_{\text{sm}}$-actin isoform, a higher proportion of the total actin exists in the supernatant fraction. Neither of the highest expressing clones transfected with the wild-type human $\beta$-actin or $\gamma$-actin showed any significant difference from C2 cells in the distribution of actin between the soluble and particulate pool (data not shown). By assaying cell extracts for G-actin using the DNase I inhibition assay (Table I), we also confirmed that cell lines expressing a higher amount of the $\beta_{\text{sm}}$-actin contained a higher amount of unassembled actin. The increase in G-actin measured by the DNase I assay is not as large as the increase observed in the supernatant actin pool, perhaps indicating that some of the increase in non-sedimentable actin results from aggregates or oligomers that do not inhibit DNase I stoichiometrically. The percentage increase in actin in the soluble pool roughly parallels the decline observed in ADF expression, suggesting that ADF expression may be sensitive to feedback regulation as a result of increased monomer and/or decreased polymer.

To explore this hypothesis further, we examined the effect of the cell-permeable actin depolymerizing agent, Lat A, on the synthesis of ADF. By binding to monomeric actin (47), Lat A increases monomeric actin pools in Swiss 3T3 and HeLa cells, a change which is accompanied by a decrease in actin synthesis (8). C2 cells rounded up within 20 min of Lat A addition to 5 μM. Unassembled actin in Triton X-100-soluble extracts of C2 cells treated with this same concentration of Lat A increased from 34 ± 3% of total actin to 58 ± 4% (constant from 6 to 18 h after Lat A addition), a shift comparable with that observed previously (8). Over the 18-h time course of Lat A treatment, ADF and cofilin did not change significantly as a percent of total protein (not shown). However, even within 6 h of Lat A treatment, nearly all of the ADF and cofilin became phosphorylated (Fig. 6). The phosphorylated forms of ADF and cofilin are inactive in binding actin (45, 48). Thus, the immediate response of C2 cells to an increase in unassembled actin is to inactivate both ADF and cofilin.

The amounts of radioactivity incorporated into ADF, cofilin, and actin by pulse labeling before and after Lat A treatment were determined by PhosphorImaging and compared with 19 randomly selected protein spots. Exposure of C2 cells to Lat A for greater than 12 h caused a significant decline in overall protein synthesis. As shown in Fig. 7, cofilin synthesis closely follows the average levels of protein synthesis during the first 12 h. However, ADF and actin synthesis decrease much more rapidly following Lat A addition, paralleling the increase in unassembled actin in Lat A-treated cells. These results demonstrate that the expression of ADF, but not cofilin, is sensitive to the utilization of actin by the cell.

![ADF has the ability to bind to both the unassembled mutant $\beta_{\text{sm}}$-actin and normal actin from transfected C2C12 cells. Western blots of both ADF immunoprecipitates (A) and actin bound to ADF resin from lysates of transfected C2 cells (B) showed ratios of $\beta_{\text{sm}}$-actin to normal actin similar to those found in the whole cell lysate.](image)

**Table I**

| Clone | Fraction | $\beta$-Actin | $\beta_{\text{sm}}$-actin | $\gamma$-Actin | Percent of actin in supernatant | Percent increase in supernatant actin | Percent G-actin increase (DNase I assay) | Percent decrease in ADF |
|-------|----------|---------------|----------------|--------------|-------------------------------|-------------------------------------|-------------------------------------|----------------------|
| C2    | sup      | 21            | 0             | 8            | 33                            | 0                                   | 0                                   | 0                    |
|       | pel      | 38            | 0             | 20           |                               |                                     |                                     |                      |
| 547   | sup      | 23            | 1.5           | 19           | 42                            | 27                                  | 2 ± 8                               | 16                   |
|       | pel      | 35            | 4.5           | 20           |                               |                                     |                                     |                      |
| 572   | sup      | 11            | 21            | 10           | 45                            | 36                                  | 38 ± 9                              | 55                   |
|       | pel      | 18            | 22            | 12           |                               |                                     |                                     |                      |
| 522   | sup      | 8             | 34            | 8            | 58                            | 76                                  | 56 ± 11                             | 76                   |
|       | pel      | 9             | 22            | 7            |                               |                                     |                                     |                      |
DISCUSSION

The ability of an actin mutant to specifically down-regulate ADF suggests the existence of a unique regulatory pathway linking ADF with an unknown aspect of actin function. The specificity of this pathway is highlighted by the finding that β-sm-actin transfections parallel each other in all aspects except ADF regulation (6, 10, 11, 31). We doubt, however, that ADF is responding to the decreased levels of normal actin per se because the binding studies suggest that ADF can equally bind normal and β-sm-actin. Unlike γ-actin, the mutant β-sm-actin cannot form normal actin filaments but rather assembles into ribbon-like structures (22). ADF regulation may therefore be responding to either the decrease in F-actin available for binding or to the increase in the G-actin pool. The ability of Lat A to induce decreased synthesis of ADF but not cofilin confirms that the G-/F-actin ratio can certainly regulate ADF metabolism.

The difference in regulation of expression of ADF and cofilin is of considerable interest because they are members of the same family of 18.5 kDa, calcium-independent, pH-sensitive F-actin-binding/depolymerizing and G-actin-sequestering proteins (26, 27, 40, 49–52). Both proteins have identical regulatory sites for phosphorylation (48, 53) and have been identified as proteins that undergo rapid dephosphorylation in response to external stimuli which result in changes in cytoskeletal organization and assembly (54–57). The results presented here demonstrate that both proteins respond identically to post-translational regulation in response to increased monomeric actin pools. This is consistent with a model in which elevated G-actin initially inactivates both ADF and cofilin by phosphorylation. If this G-actin elevation persists, a second level of control of ADF activity is necessary for regulating actin assembly and morphology, do not affect the expression of other microfilament components and can send signals to, but does not respond to, the expression of these other gene products. Recent studies in which the single ADF/cofilin gene product in Dictostelium discoideum was overexpressed suggest that this is not true (68). A 7-fold increase was achieved in Dictostelium cofilin expression, but this was accompanied by a 3-fold increase in actin expression, suggesting that for certain actin-binding proteins, especially those that are involved in setting or maintaining the monomeric actin pool, compensatory changes in actin synthesis are triggered. This suggests that it is direct alteration in actin metabolism that may regulate actin and other actin-binding proteins.

What then is the biological function of ADF? These data certainly do not support a role as a simple actin monomer sequestering protein. Its biosynthetic regulation is consistent with what one would expect for a protein whose function is to set and maintain the G-/F-actin ratio. Indeed, it is the proteins of the ADF/cofilin family that are necessary to turn over the actin in the tails of Listeria monocytogenes, the intracellular bacterium that utilizes actin assembly to propel itself around...
the cytoplasm of infected cells (69). We propose that through both posttranslational and biosynthetic regulation, ADF plays a role in regulating actin polymer levels in C2 cells.

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