Kinetic Analysis of the Interaction of Actin-depolymerizing Factor (ADF)/Cofilin with G- and F-Actins

COMPARISON OF PLANT AND HUMAN ADFs AND EFFECT OF PHOSPHORYLATION*

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The thermodynamics and kinetics of actin interaction with Arabidopsis thaliana actin-depolymerizing factor (ADF), human ADF, and S6D mutant ADF, protein mimicking phosphorylated (inactive) ADF are examined comparatively. ADFs interact with ADP·G-actin in rapid equilibrium (k + = 155 μM⁻¹·s⁻¹ and k - = 16 s⁻¹ at 4 °C under physiological ionic conditions). The kinetics of interaction of plant and human ADFs with F-actin are slower and exhibit kinetic cooperativity, consistent with a scheme in which the initial binding of ADF to two adjacent subunits of the filament nucleates a structural change that propagates along the filament, allowing faster binding of ADF in a “zipper” mode. ADF binds in a non-cooperative faster process to gelsolin-capped filaments or to subtilisin-cleaved F-actin, which are structurally different from standard filaments (Orlova, A., Prochniewicz, E., and Edelman, E. H. (1985) J. Mol. Biol. 245, 598–607). In contrast, the binding of phalloidin to F-actin cooperatively inhibits its interaction with ADF. The ADF-facilitated nucleation of ADP-actin self-assembly indicates that ADF stabilizes lateral interactions in the filament. Plant and human ADFs cause only partial depolymerization of F-actin at pH 6, consistent with identical functions in enhancing F-actin dynamics. Phosphorylation does not affect ADF activity per se, but decreases its affinity for actin by 20-fold.

A large body of evidence supports the view that the rapid turnover of actin filaments drives actin-based motility processes such as the forward movement of the leading edge of the lamellipodium of locomoting cells, the propulsive movement of Listeria monocytogenes, the movement of cortical actin patches in yeast, or the extension of the growth cone (1). Filaments turn over via a treadmilling mechanism, whereby the steady growth of barbed ends is fed by the subunits depolymerizing from the pointed ends (2, 3). While barbed end growth, which provides the motile force (4), is restricted to specialized regions of the cell such as the leading edge (5), depolymerization may occur from all pointed ends in the cell medium. The observed rates of actin-based movement fall in the range 1–20 μm/min, corresponding to treadmilling rates of 7–130 subunits/filament, i.e. 1–2 orders of magnitude higher than the treadmilling rate measured in vitro in solutions of pure actin (6).

Actin-binding proteins of the ADF/cofilin family have recently been demonstrated to enhance the treadmilling of F-actin in vitro by 25-fold and consistently to increase the rate of Listeria propulsion in platelet extracts (7). We proposed (7) that these in vitro properties of ADF accounted for the enhancement of motility of Dictyostelium discoideum (8) due to ADF overexpression and for its high level of expression in early development (9). Consistently, ADF was shown to be responsible for the high rate of filament turnover in yeast (10).

It was initially thought that ADF depolymerized F-actin rapidly due to a severing activity (11–14). Severing of the filaments was thought to create a large number of uncapped barbed ends, known to depolymerize rapidly. It is worth stressing that the above reasoning is incorrect in the cellular context of the steady state of actin assembly, in which the concentration of ATP-G-actin always lies between the critical concentrations of the barbed and pointed ends. In such a situation, uncapped barbed ends that would putatively be created by severing of the filaments would immediately grow and not depolymerize. Recent data show that the ADF-induced enhancement of treadmilling is not due to severing, but to an increase in the rate of depolymerization from the pointed ends, which is the rate-limiting step in the treadmilling cycle (7, 15). This function of ADF is mediated by its specific binding to ADP-bound G- and F-actins, thereby participating in actin filament assembly while changing the assembly rate parameters in an end-specific fashion.

A unique feature of all ADFs (except maybe yeast ADF) is their ability to be activated by rapid dephosphorylation in response to diverse stimuli that lead to changes in actin dynamics (see Ref. 16 for a review). The phosphorylated serine 3 in vertebrate ADF and serine 6 in plant ADF (17) resides in the N-terminal region of the molecule. The three-dimensional structure of unphosphorylated ADF from different sources has been solved using NMR (18) or x-ray diffraction (19, 20) and shows a remarkable similarity to gelsolin segment-1. An extensive mutagenesis study, however, demonstrated that yeast cofilin interacts with actin in a manner quite
different from gelsolin segment-1 (21), consistent with the different functions of these two proteins. Image reconstruction of frozen hydrated specimens of filaments decorated with ADF indicates that ADF binds cooperatively to F-actin while changing the helical periodicity of the filament (14).

To analyze and simulate the effects of ADF on actin dynamics quantitatively, it is necessary to have access to the rate parameters of ADF interaction with both G- and F-actins. This rapid kinetic study is carried out here using human ADF and plant ADF (Arabidopsis thaliana ADF1) on a comparative basis. Indeed, although structural and functional evidence indicates that all ADFs have identical regulatory properties (7, 18-21), quantitative differences may exist between ADFs from different species, which may introduce important differences in their function. For instance, human ADF has been reported to cause total depolymerization of F-actin at pH 8.0 (13), whereas plant ADF causes only partial depolymerization at all pH values (7). The effect of phosphorylation of ADF on its function is also investigated here using the serine to aspartate mutation of serine 3 in A. thaliana ADF1 to mimic phosphorylation. The binding parameters and the activity of the S6D mutant of ADF1 have been characterized to determine whether phosphorylation affects the activity of ADF1, or its binding to actin.

Kinetics also offer the opportunity to elucidate the mechanism by which ADF changes the structure of the filament. Kinetic analysis of the binding of ADF to filaments maintained in different structural states by drugs (phalloidin), regulatory proteins (gelsolin), or chemical modification of subdomain 2 provides further insight in the structure of ADF-F-actin.

**Materials and Methods**

**Proteins—**Actin was purified from rabbit muscle and isolated as CaATP-G-actin through Sephadex G-200 chromatography in G buffer (5 mM Tris-Cl, pH 7.5, 0.1 mM CaCl₂, 0.2 mM dithiothreitol, 0.2 mM ATP, and 0.01% NaN₃). Procedures to prepare NBD-labeled actin, MgATP-G-actin, and MgADP-G-actin were as described previously (7). Subtilisin-cleaved actin (22) was prepared by incubating CaATP-G-actin (20 μM) in G buffer with subtilisin Carlsberg (Sigma) at a 1:1500 (w/w) subtilisin/actin ratio for 45 min at 20°C. The reaction was stopped by addition of 1 mM phenylmethylsulfonyl fluoride.

Human ADF and A. thaliana ADF1, were recombinant proteins expressed in Escherichia coli using the pET vector and were purified by DEAE-cellulose and SP-Trisacryl chromatography as described (7). The S6D mutant of ADF1 was produced and purified in the same way. Gelsolin from human plasma was a gift from Dr. Yukio Doi. Protein concentrations were determined spectrophotometrically using extinction coefficients of 0.617 mg⁻¹ cm⁻² for actin at 290 nm, 0.89 mg⁻¹ cm⁻² for wild-type and S6D mutant ADFs at 278 nm (7), and 0.64 mg⁻¹ cm⁻² for human ADF (13).

**Static Fluorescence Measurements—**The change in fluorescence of NBD-labeled G-actin was used to monitor the binding of ADF to G-actin under a variety ofionic conditions, as described previously (7). It was observed that human ADF, in contrast to plant ADF, did not modify the fluorescence of NBD-labeled actin to an appreciable extent. Therefore, in most cases, the equilibrium dissociation constant for the binding of human ADF to G-actin was derived from competition studies, in which the binding of ADF was measured in the presence of different known amounts of human ADF or by displacing actin-bound ADF, by addition of increasing amounts of human ADF.

Fluorescence titration curves were analyzed as follows to derive the equilibrium dissociation constant of the human ADF-G-actin complex. The observed fluorescence (F) can be written as follows (Equation 1),

\[
F = f_0[A] + f_1[AX] + f_2[AY]
\]

where [A], [AX], and [AY] represent the concentrations of free G-actin, ADF-G-actin, and human ADF-G-actin complexes, respectively, and \(f_0, f_1, \text{ and } f_2\) represent the specific fluorescence of actin in the respective species.

**Equation 1 can be written as follows (Equation 2),

\[
F = \frac{f_0[A] + f_1[AX] + f_2[AY]}{1 + \frac{[X]}{K_x} + \frac{[Y]}{K_y}}
\]

where \(K_x\) and \(K_y\) are the equilibrium dissociation constants for the ADF-G-actin (AX) and human ADF-G-actin (AY) complexes, respectively. Theoretical curves were computer-generated using Equation 2 and calculated values of the concentrations of free ADF, (X) and free human ADF (Y) as follows. Combining the laws of mass action and mass conservation leads to Equation 3.

\[
Y^2 + Y \cdot \left( [A] - [Y] + K_y \cdot \left( 1 + \frac{Y}{K_y} \right) \right)
- Y \cdot K_y \cdot \left( 1 + \frac{X}{K_x} \right) = 0
\]

The concentration of free human ADF (Y) was calculated as a function of the concentration of free ADF, (X) by solving Equation 3. Incremented values of [X] were computer-generated, from which corresponding values of [Y] were obtained, for the given values of total human ADF concentration ([Y]₀). Then the total concentration of ADF (X[₀]) was calculated as follows (Equation 4).

\[
X[₀] = [X] + [AX]
\]

The calculated value of F was plotted as a function of [X]₀. Values of \(f_0, f_1, \text{ and } f_2\) used in the calculation of F were experimentally determined. The value of \(K_y\) was first easily derived from analysis of the titration curve of G-actin by ADF1, in the absence of human ADF. Its value was used in the calculation of F, [X], and [X]₀. The value of \(K_y\) was then adjusted manually to obtain the best global fit to all the titration curves of G-actin by ADF at different concentrations [X]₀ of human ADF.

**Kinetic Measurements—**The kinetics of binding of ADF1 to G-actin were monitored by the quenching of NBD-labeled G-actin fluorescence using a stopped-flow apparatus (DX.17 MV, Applied Photophysics Ltd.). NBD-labeled G-actin (in either MgATP- or MgADP-bound form) in syringe A was mixed with ADF, present at different concentrations in syringe B. Experiments were carried out at low ionic strength (G buffer) and at physiological ionic strength (0.1 M KCl and 1 mM MgCl₂ added to G buffer). The change in NBD fluorescence was recorded (λex = 475 nm). A filter cutting the excitation light (0% transmission at λ = 515 nm and 65 and 80% transmission at 530 and 540 nm, respectively) was placed on the emission beam. Dissociation of ADF1 from G-actin was similarly monitored by displacing bound ADF1 from the preformed NBD-labeled ADF1-G-actin complex (syringe A) by an excess of unlabeled G-actin (syringe B). Dissociation of human ADF from G-actin was monitored by displacing bound human ADF from the preformed NBD-labeled ADF1-G-actin complex by an excess of ADF1.

The kinetics of binding of human and ADFs to F-actin were monitored in the stopped-flow apparatus using both light scattering and NBD fluorescence. The changes in light scattering and fluorescence were recorded using the same solutions of F-actin and ADFs. Light scattering was monitored at 320 nm, with a 270-μs electronic attenuation filter to minimize the noise. Superimposable traces recorded from a minimum of six consecutive shots were averaged and used for kinetic analysis. The changes in NBD fluorescence were recorded as described above. Because fluorescence data are less noisy than light scattering, three superimposable traces coming from consecutive shots were averaged. The kinetics of dissociation of ADF from F-actin were monitored in a Spex spectrophuorometer by displacing ADF bound to NBD-labeled F-actin (1–2 μM) by an excess of unlabeled F-actin (10–50 μM).

**Sedimentation Assay—**The binding of ADFs and S6D mutant ADF1 to F-actin and the extent of depolymerization linked to ADF binding were measured by SDS-polyacrylamide gel electrophoresis analysis of the pellets and supernatants of samples containing different amounts of F-actin and ADF (7). Experiments were carried out at pH 6.5 and 8.2. The steady-state concentration of monomeric actin and ADF present in the supernatant was estimated by scanning Coomasie Blue-stained gels using an Arcus II scanner (AGFA Corp., Orangeburg, NY) and
Kinetics of ADF Interaction with Actin

Table I

| Species                        | Ionic conditions        | NBD-actin fluorescence | $K_D$ (μM) |
|--------------------------------|-------------------------|------------------------|------------|
| ADP$_1$ATP-G-actin             | Low ion strength buffer | 0.68 ± 0.02            | 0.05       |
| hADF$_1$ATP-G-actin            | (G buffer), pH 7.6      | 1.1 ± 0.03             | 0.07       |
| S6D ADF$_1$ATP-G-actin         |                         | 0.75 ± 0.03            | 0.47       |
| hADF$_1$ADP-G-actin            |                         | 0.58 ± 0.03            | 0.03       |
| S6D ADF$_1$ADP-G-actin         |                         | 1 ± 0.03               | 0.02       |
| hADF$_1$ADP$_2$G-actin         |                         | 0.72 ± 0.03            | 0.04       |
| hADF$_1$ATP$_2$G-actin         | Physiological ion       | 0.72 ± 0.03            | 7          |
| hADF$_1$ATP$_2$G-actin         | strength buffer         | 1.25 ± 0.05            | 6          |
| S6D ADF$_1$ATP$_2$G-actin      |                         | 0.75 ± 0.03 >20        |            |
| hADF$_1$ADP$_2$G-actin         |                         | 0.4 ± 0.05             | 0.19       |
| S6D ADF$_1$ADP$_2$G-actin      |                         | 1.0 ± 0.1              | 0.09       |
| hADF$_1$ADP$_2$G-actin         |                         | 0.8 ± 0.1              | 1.5        |

* hADF$_1$, human ADF.

Values of $K_D$ for complexes of G-actin with ADP$_1$ and S6D mutant ADF$_1$ were derived from fluorescence titration curves of NBD-labeled G-actin. Values of $K_D$ for complexes of G-actin with human ADF were derived from analysis of the competition between ADP$_1$ and human ADF as described under "Materials and Methods." By convention, a value of 1.0 is attributed to the specific fluorescence of NBD-labeled G-actin under each ionic condition.

Comparing with actin and ADF standards using the National Institutes of Health Image analysis program.

ATPase Measurements—Measurements of the steady-state hydrolysis of ATP resulting from treadmilling of F-actin were carried out in the presence of different concentrations of wild-type or mutant ADF proteins as described (7). Actin (15 μM) was polymerized in the presence of [γ-32P]ATP. When steady state was reached, the solution was split into several samples supplemented with different amounts of ADF. The hydrolysis of ATP was monitored at 50-min intervals over a period of 6 h.

Results

Equilibrium Binding of Human ADF and Arabidopsis Wild-type and S6D Mutant ADF to G-actin—A. thaliana ADF$_1$ causes a 35% quenching of NBD-labeled actin fluorescence upon binding to G-actin (7). In contrast, the binding of human ADF very little affected the fluorescence of NBD-labeled G-actin. Therefore, the equilibrium dissociation constant for human ADF binding to G-actin was derived from fluorescence titration curves of NBD-labeled G-actin by Arabidopsis ADF$_1$ in the presence of human ADF acting as a competitive ligand. Examples are shown in Fig. 1. Data were fitted as developed under "Materials and Methods" to derive the values of the equilibrium dissociation constants. All data obtained under different ionic and actin-bound nucleotide conditions are summarized in Table I. The two ADF variants appear to have very similar affinities for G-actin under a variety of conditions. Human ADF, like plant ADF, shows high affinity ($10^{-8}$ M$^{-1}$) for ADP-G-actin and very low affinity ($10^{-5}$ M$^{-1}$) for ATP-G-actin under physiological ionic conditions. This result is at variance with previous reports (12, 13) in which the shift in critical concentration plots induced by ADF was interpreted as evidence for high affinity binding of ADF to ATP-G-actin. The reason for this discrepancy will be clarified under “Discussion.”

The binding of the S6D mutant ADF$_1$ protein was measured in a similar fashion. S6D mutant ADF$_1$ affected the fluorescence of NBD-labeled G-actin like the wild-type protein, indicating that the interfaces of the mutant and wild-type proteins with G-actin were the same, but showed a 20-fold lower affinity (Table I).

Kinetics of Binding of ADF to G-actin—The kinetics of association of ADF$_1$ with NBD-labeled G-actin were monitored by fluorescence in the stopped-flow apparatus. At all ionic strengths, the association of ADF$_1$ with either ATP-G-actin or ADP-G-actin was extremely rapid and could be monitored by lowering the temperature to 4 °C. At physiological ionic strength and at 4 °C, the time course of the decrease in NBD fluorescence shown in Fig. 2 reflected the reversible bimolecular association of ADF$_1$ with actin, with association and dissociation rate constants of 155 ± 10 μM$^{-1}$ s$^{-1}$ and 16 ± 10 s$^{-1}$, respectively. The dissociation rate constant could be directly determined with a greater accuracy by displacing ADF$_1$ bound to NBD-labeled ADP-G-actin by an excess of unlabeled ADP-G-actin. The resulting increase in fluorescence is kinetically limited by the dissociation of ADF$_1$ from NBD-labeled G-actin. A value of 12 s$^{-1}$ was therefore obtained for the dissociation rate constant of ADF$_1$. Similarly, the rate constant for dissociation of human ADF from ADP-G-actin was derived from the exponential decrease in fluorescence recorded upon displacing human ADF bound to ADP-G-actin by a large excess of ADF$_1$. A value of 13 s$^{-1}$ was obtained for the dissociation rate constant of human ADF. In conclusion, under physiological conditions, plant and human ADFs bind ADP-G-actin with high affinity and in very rapid association/dissociation equilibrium.

fig. 1. Fluorescence titration of NBD-labeled G-actin by plant and human ADFs. a, binding to ATP-G-actin. Arabidopsis ADF$_1$, at the indicated concentrations was added to fully NBD-labeled ATP-G-actin (1 μM) in the absence (closed squares) and presence of 1.1 μM (open squares) or 2.2 μM (open circles) human ADF in G buffer at pH 7.6. Closed circles represent the titration curve of ATP-G-actin by human ADF. Continuous lines were calculated using $K_D$ values of 0.07 and 0.65 μM for the complexes of ATP-G-actin with plant and human ADFs, respectively, in a competitive binding scheme and normalized specific fluorescence of 1, 0.61, and 1.16 for G-actin, ADF$_1$G-actin, and human ADFG-actin, respectively. b, binding to ADP-G-actin. Arabidopsis ADF$_1$ was added to 1 μM NBD-labeled ADP-G-actin in the absence (closed circles) and presence of 1 μM (open squares) or 2 μM (open squares) human ADF in G buffer containing ADP at pH 7.6. The binding of human ADF does not affect the fluorescence of ADP-G-actin. Curves were calculated using $K_D$ values of 0.03 and 0.02 μM for the complexes of ADP-G-actin with plant and human ADFs, respectively. α, arbitrary units.
Kinetics of Binding of ADF to F-actin—The kinetics of association of plant and human ADFs with F-actin were monitored in the stopped-flow apparatus by both NBD fluorescence and light scattering in ATP-containing F buffer (G buffer supplemented with 0.1 M KCl and 1 mM MgCl₂) at 20 °C and pH 7.6. While the fluorescence of NBD-labeled G-actin was unaffected by human ADF, the fluorescence of NBD-labeled F-actin was quenched by human ADF, as previously observed with Arabidopsis ADF₁ in static measurements (7). Fig. 3 (a and b) shows that the quenching of fluorescence temporally correlates with an increase in light scattering, which reflects the 42% increase in mass per unit length of the filament associated with ADF (18 kDa) binding to each F-actin subunit (42 kDa). The initial increase in light scattering was followed by a slower decrease (Fig. 3c), which, as previously observed with ADF₁ (7), reflects the partial depolymerization of ADF-F-actin, and relaxation toward a new steady state in which ADF is bound in part to ADF-G-actin and in part to F-actin. Therefore, the amount of ADF-F-actin at the end of the rapid phase is greater than at the end of the slow phase. The extent of fluorescence decrease reached at the end of the rapid phase (Fig. 3d) truly represents the binding of ADF to F-actin before the partial depolymerization of ADF-F-actin into ADF-ADP-G-actin has occurred. This curve therefore does not exhibit the strong sigmoidal shape displayed by curves derived from sedimentation data, when the amount of ADF-F-actin was plotted versus the concentration of ADF in the supernatant, misleadingly assumed to represent the concentration of free ADF (14). In view of the present and previous (7) data, this strong sigmoidal behavior results from the binding of ADF to both G- and F-actins at steady state. The extent of depolymerization was greater at pH 8 than at pH 6.8 (7); otherwise, the overshoot kinetic curves observed in light scattering were similar at all pH values.

Fig. 4a shows that human ADF bound more slowly to F-actin than Arabidopsis ADF₁. The extent of fluorescence quenching was also slightly lower for human ADF. For the two ADF species, the time courses of the increase in light scattering and of quenching of NBD-labeled F-actin fluorescence were not consistent with a simple bimolecular reaction, but showed an acceleration following a lag phase, indicating that ADF bound to F-actin in a kinetically cooperative fashion (Fig. 4b). This behavior suggests that the binding of the first ADF molecules to a bare filament is accompanied by a large cooperative structural change of this filament, allowing the faster subsequent binding of ADF.

To get more insight into the mechanism by which ADF modifies the structure of F-actin, attempts were made to elaborate...
The kinetics of ADF binding to F-actin were simulated within the above scheme by integration of Equations 5 and 6 using Excel (Microsoft). A good fit to the whole series of curves could be obtained only for \( n = 2 \). Higher values of \( n \) were unsatisfactory, independent of the values of \( k_n \) and \( k_s \). Calculated curves (Fig. 4b) represented the best fit to the data, using the experimental values of \( F_0 \) and ADF\(_0 \) and values of 0.037 and 130 \( \mu M^{-1} s^{-1} \) for \( k_n \) and \( k_s \), respectively.

The kinetic cooperativity in the binding of ADF to F-actin results in equilibrium binding curves (expressed as a function of total ligand) that differ, in the 50–90% saturation range, from the behavior expected for michaelian binding. The data points above 50% saturation (Fig. 3d) fall above the dashed line representing michaelian binding of ADF to F-actin with an equilibrium dissociation constant of 0.3 \( \mu M \) (7), consistent with cooperative binding.

The rate of ADF binding to F-actin was pH-dependent. Kinetic cooperativity was observed at all pH values; however, the binding was ~4-fold slower at pH 8.2 than at pH 7.2.

The kinetics of ADF binding to Ca\(^{2+}\)-F-actin or Mg\(^{2+}\)-F-actin were similarly cooperative and not appreciably different in rate. The only difference was that the quenching of fluorescence of NBD-labeled F-actin was 11.5% greater when Mg\(^{2+}\) was bound than when Ca\(^{2+}\) was bound to actin.

Internal cooperativity in the structure of F-actin has already been observed (23–27). Both the structure and the flexural rigidity of the actin filament are affected by the nature of the bound nucleotide or ligands like phalloidin (28–30). A cooperative change in structure propagating over long distances along the filament has been observed when filaments are capped by gelsolin or assembled from actin proteolytically cleaved in subdomain 2 (27, 28). This structural change is visualized by a bridge of density connecting the two strands of the long pitch helix. The fact that ADF does not bind to filaments stabilized by phalloidin or BeF\(_3\) (7) and promotes a structural change in the filament when it binds ADF-F-actin prompted us to examine the kinetics of ADF binding to filaments capped by gelsolin or assembled from subtilisin-cleaved actin. Fig. 5a shows that the rate of ADF binding to capped filaments increased with the gelsolin/actin ratio. The effect of gelsolin could be detected at a 1:200 ratio to actin. At a gelsolin/actin ratio of 1:20, the lag time disappeared, and ADF bound to capped filaments with a rapid initial rate. At gelsolin/actin ratios of 1:20 and 1:10, the quenching of NBD-labeled F-actin fluorescence due to ADF binding was reduced by 10 and 20%, respectively, indicating that ADF did not bind to the two terminal F-actin subunits interacting with gelsolin at the barbed end, which both exhibited the fluorescence of F-actin. These results suggest that, in agreement with Egelman and coworkers (27), the structure of the filaments is affected by gelsolin in such a way that ADF binding is facilitated and does not require a nucleation step. However, our data indicate that the structural change of the filament induced by gelsolin propagates over 10–20 rather than several hundred subunits. Similarly, when loop 45–52 in actin subdomain 2 was cleaved by subtilisin, ADF bound much faster to F-actin, with a marked reduction of the lag time (Fig. 5b).

**Fig. 4. ADF binds to F-actin with a strong kinetic cooperativity.** a, comparison of plant and human ADF binding kinetics. NBD-labeled F-actin (2.5 \( \mu M \)) was mixed with Arabidopsis ADF\(_0\), (thick lines) or human ADF (thin lines) at 2.5, 5, 7.5, 10, 12.5, 15, and 20 \( \mu M \) (top to bottom curves). Note the difference in the association rates and extent of fluorescence quenching displayed by the two ADFs. Conditions were as described for Fig. 3. b, fit of the proposed model to the kinetic data for ADF\(_1\) binding to F-actin (from a) within the scheme given under “Results,” using the following values of the rate parameters: \( n = 2 \), \( k_n = 0.037 \mu M^{-1} s^{-1} \), and \( k_s = 130 \mu M^{-1} s^{-1} \). a.u., arbitrary units.

A model quantitatively accounting for the time courses of ADF binding to F-actin shown in Fig. 4b. A simple model in which the binding site consisted of two neighboring F-actin subunits (\( F_2 \)), binding two consecutive ADF molecules with very different rate constants, was first envisaged. It was possible to find sets of rate parameters that generated calculated curves able to fit some of the experimental curves, but this model failed to provide a satisfactory global fit to a series of time courses covering a broad range of ADF concentrations. The curves calculated using this model did not reconstitute the highly cooperative concentration dependence exhibited by the experimental kinetic curves.

A satisfactory fit to the complete series of kinetic curves was provided by a model within which ADF binds to a bare filament in two consecutive steps. In an initial slow “nucleation” step, the cooperative association of ADF with a critical number \((n)\) of neighboring F-actin subunits (forming a “nucleus”) changes locally the structure of the filament, thus allowing faster subsequent binding of ADF along the filament in a “zipper-like” elongation process. This model is described by the following scheme: \( F + n \text{ADF} \rightarrow F_n \text{ (nucleation)} \) and \( F_n + \text{ADF} \rightarrow F_n + \text{ADF} \text{ (elongation)} \), leading to Equations 5 and 6,

\[
\frac{d[F]}{dt} = k_n \cdot [F] \cdot [\text{ADF}]^n \quad (\text{Eq. 5})
\]

\[
\frac{d[\text{ADF} \cdot F]}{dt} = k_n \cdot [F_n] \cdot ([F]/[F_0]) \cdot [\text{ADF}] \quad (\text{Eq. 6})
\]

where \([F_0]\) and \([\text{ADF}_0]\) represent the total concentrations of F-actin subunits and ADF, respectively, and \([F_n]\) is the concentration of “nucleating” sites on F-actin. For simplicity, it is assumed that \([F_n] \ll [F]_0\) and that the reactions are irreversible. The change in fluorescence is proportional to the concentration of ADF-F-actin. In Equation 6, the factor \([F]/[F_0]\) is included to express the fact that the concentration of nuclei \([F_n]\) decreases as stretches of F-actin are entirely decorated by ADF in the progress of the binding reaction.

Kinetics of ADF Interaction with Actin
Phalloidin inhibited ADF binding to F-actin cooperatively in a substoichiometric fashion (Fig. 6). Over 90% of the F-actin subunits did not bind ADF at a phalloidin/actin ratio of 1:3, indicating that binding of phalloidin to one subunit cooperatively modifies the local structure of the filament, making a sequence of three subunits inaccessible to ADF. The binding of ADF to accessible sites was also much slower than to bare filaments.

To understand how the interaction of ADF with actin affects the dynamics of actin filaments, it is important to know the rate of dissociation of ADF from F-actin. The dissociation of ADF from F-actin was monitored by the increase in fluorescence linked to the displacement of ADF bound to NBD-labeled F-actin by an excess of unlabeled F-actin. The rate constant of the observed first-order process had a value of 0.035 s⁻¹, independent of the initial saturation level of NBD-labeled F-actin by ADF. Alternatively, ADF dissociation from NBD-labeled F-actin could be elicited by phalloidin since ADF and phalloidin bind to F-actin in a mutually exclusive fashion (7). The observed fluorescence increase was a monoexponential process at a high concentration of phalloidin. The first-order rate constant reached a finite limit of 0.1 s⁻¹. This value is higher than the one derived from the chase experiment described above, indicating that phalloidin first binds to ADF-F-actin, and then ADF dissociates from the ternary ADF-F-actin-phalloidin complex.

Human ADF Causes Partial Depolymerization of F-actin in a pH-dependent Fashion, Qualitatively Similar to Plant ADF—Chick or human ADF has been reported to cause complete depolymerization of F-actin at pH 8.0 (12, 13). This result suggested that ADF was a G-actin-sequestering protein, a conclusion at variance with the ones derived from a more recent study of plant ADF (7) that shows that ADF interacts with both F- and G-actins. To understand whether vertebrate ADF might have different properties from plant ADF, analysis of the ADF-induced depolymerization at different pH values was carried out using a sedimentation assay. The experiments shown in Fig. 7 were conducted either by adding increasing amounts of human ADF to F-actin at a given concentration (20 μM) or by adding increasing amounts of F-actin to human ADF maintained at a constant concentration (30 μM). Human ADF caused a more extensive depolymerization of F-actin than plant ADF at all pH values; nonetheless, the depolymerization always occurred to a limited extent. At pH 6.5, a maximum of 2 μM actin was depolymerized by human ADF, a concentration 2-fold higher than with Arabidopsis ADF (7). At pH 8.2, only 6–7 μM actin was depolymerized by human ADF (versus 2.5 μM actin for plant ADF). This result is at variance with reports by Hawkins et al. (12) and Hayden et al. (13), but is consistent with the polymerization properties of ADF-ADP-actin, as shown below.

ADPG-actin polymerizes reversibly with a critical concentration of 1.5 μM under physiological ionic conditions (31). The
nucleation of ADF-actin is energetically very difficult (32, 33). In contrast, the nucleation of the ADF-ADF-actin complex to form ADF-decorated filaments appeared extremely easy (Fig. 8, inset). The assembly process, monitored turbidimetrically, displayed no detectable lag, indicating that nuclei formed rapidly. A critical concentration plot was derived from measurements of the extent of turbidity change at the end of the reaction (Fig. 8). The critical concentrations for assembly of ADF-ADF-F-actin from the ADF-ADF-G-actin complex were 3.5 and 8 μM for plant and human ADFs, respectively, at pH 8 under physiological ionic conditions. These data demonstrate that it is not theoretically possible to depolymerize more than 8 μM actin with vertebrate ADF, in agreement with the experimental evidence displayed in Fig. 7.

**Effect of Phosphorylation on ADF Function and Interaction with F-actin**—The binding of S6D mutant ADF1 to F-actin was examined by the fluorescence and sedimentation assays. The S6D mutant ADF1-elicted depolymerization of F-actin was very weak in the concentration range investigated (0–20 μM). Fluorescence titration curves of NBD-labeled F-actin by S6D mutant ADF1 were consistent with an order of magnitude lower affinity of S6D mutant ADF1 than the wild-type protein for actin (data not shown). S6D mutant ADF1 bound NBD-labeled F-actin in a kinetically cooperative fashion like the wild-type protein. The binding process was 10-fold slower than with wild-type ADF1. Specifically, when 3 μM NBD-labeled F-actin was reacted with 12 μM ADF1, the lag time was 18 ms, and the t1/2 of the reaction was 60 ms. When it was reacted with 12 μM S6D mutant ADF1, the lag time was 200 ms, and the t1/2 was 0.72 s (data not shown).

Although S6D mutant ADF1 binds G- and F-actins with low affinity, the question should be raised whether the biological activity is affected by the mutation. Fig. 9 shows that S6D mutant ADF1 enhances the treadmilling of actin filaments as all ADFs do (7); however, the effect develops in the higher range of protein concentration at which the mutant protein binds to F- and G-actins. In conclusion, the strength of binding of ADF is weakened by phosphorylation, but the mechanism of binding remains unchanged. In addition, once bound to actin, the S6D mutant protein affects the dynamics of filaments in the same manner as the wild-type protein.

**DISCUSSION**

Human ADF and Arabidopsis ADF1, two distant variants of the ADF/cofilin family, interact with F- and G-actins and affect actin dynamics in almost identical fashion. The main conclusion of the direct binding studies using unmodified ADF is that ADF binds essentially ADP-actin with an affinity of $10^8$ M$^{-1}$ and practically not ATP-actin under cellular ionic conditions. Therefore, ADF cannot be considered as a G-actin-sequestering protein as previously thought. The high affinity of ADF for ATPG-actin, which was derived from the shift in critical concentration plots (12, 13), relied on the unverified hypothesis that ADF was forming a 1:1 complex with ATPG-actin exclusively and that this complex was in equilibrium with free ATPG-actin at the same critical concentration as in the control curve obtained in the absence of ADF. This interpretation is invalidated by the present and past (7) results showing that ADF in fact interacts with both ADP-G-actin and ADP-F-actin, which changes the dynamics of actin and incidentally affects the steady-state concentration of ATPG-actin.2

The fluorescence of NBD-labeled G-actin is not quenched upon binding of human ADF as it is upon binding of ADF1, indicating that the environment of Cys-374 on G-actin (but not

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on F-actin is not affected in the same way by the two ADFs. The mutagenesis studies of Lappalainen et al. (21) show that the G-actin-binding region in ADF/cofilin contains in particular the loop between strand β6 and helix α4. The sequence of human ADF diverges significantly from other ADFs (including ADF1) in this region, which may explain the difference in the environment of the NBD-derivatized Cys-374 of actin when different ADFs are bound.

Analysis of the properties of S6D mutant ADF1, which mimics phosphorylated ADF1, demonstrates that phosphorylation does not affect the biological activity of ADF per se or its mechanism of interaction with actin, but reduces its affinity for both G- and F-actins by 20-fold. The effect of phosphorylation then is equivalent to a 20-fold decrease in the concentration of active protein. However, once bound to actin, S6D mutant ADF1 affects actin dynamics like the unmodified protein. These results expand the conclusions derived from genetic studies (21) that indicated that phosphorylation did not grossly affect the structure of ADF.

Analysis of the kinetics of interaction of ADF with G- and F-actins is important in determining which sequence of elementary steps is going to take place and modify actin dynamics in a living cell, immediately following reception of a signal leading to ADF activation. This work shows that binding of ADF to F-actin occurs first, followed (within 2–3 min) by the establishment of a new steady state in which filaments turn over faster, with an increased accumulation of monomeric actin, consisting of ADF-ADP-G-actin, ADP-G-actin, and ATP-G-actin. ADF interacts with ADP-G-actin in rapid equilibrium. The large pool of dissociated ADF-ADP-G-actin generates an increased amount of ADP-ADF-G-actin and of ATP-G-actin as well, via nucleotide exchange. Within this view, the increased treadmilling rate is thought to be due to the increased steady-state concentration of ATP-G-actin.2 At high ADF concentration, the ADF-ADP-G-actin complex is stabilized; hence, the decline in treadmilling rate (Fig. 9) is presumably due to the association flux of ADF-ADP-G-actin to filament ends, which counteracts and limits the dissociation flux. The depolymerization induced by ADF, in ATP-containing F buffer, is limited by the polymerization of ADF-ADP-actin, which is consistently shown to occur at critical concentrations of 3.5 and 8 μM for Arabidopsis and human ADFs, respectively (Fig. 8).

ADF binds to and dissociates from F-actin relatively slowly. In particular, the rate of dissociation of ADF from F-actin is slower than the rate of depolymerization of ADF-F-actin from the pointed end at steady state. Therefore, F-actin-bound ADF does not rapidly shuttle from one filament to the other. Interestingly, ADF binds F-actin in a kinetically cooperative fashion. The binding of the first molecules of ADF to a bare filament resembles a nucleation process during which the structure of the filament is locally disrupted, thus allowing the more facile subsequent association of other ADFs with the “opened” sites along the filament. The binding then occurs linearly at a faster rate, in a zipper-like mode. There is some analogy here to the vectorial process of ATP hydrolysis on MgF-actin (34). The functional consequence of such a cooperative binding process is that the enhancement of actin dynamics by ADF (7) occurs essentially on a fiber-by-fiber basis. In other words, when ADF is added to F-actin in a 1:10 ratio, at the end of the binding process, roughly 10% (in number) of the filaments are entirely decorated by ADF, and 90% are bare, as was noticed on the electron micrographs (14). Since the ADF-decorated filaments depolymerize rapidly, ADF then visits other filaments in the population in a treadmilling-assisted process.

The assembly of ADF-F-actin from the high affinity ADF-ADP-G-actin complex is a simple case of reversible polymerization. It is remarkable that nucleation of ADF-actin is greatly facilitated by ADF, whereas the critical concentration for assembly is increased. This apparent paradox indicates that, although the actin–actin bonds involved in filament elongation are globally weakened by ADF, the energetically unfavorable actin–actin bonds involved in nucleation are strengthened by ADF. Structural and biochemical studies as well as the atomic model of the actin filament suggest that the longitudinal actin–actin bonds play a major role in filament stability (35–37) and that the lateral bonds are involved in filament nucleation (38–41). Within this view, in binding to F-actin, ADF would bridge two neighboring subunits along the small pitch helix, thus stabilizing lateral actin–actin bonds, whereas longitudinal bonds would be destabilized in ADF-decorated filaments, thus accounting for an overall increase in critical concentration. The destabilization of longitudinal bonds would account for the contorted appearance (7, 42) and increased twist (14) of ADF-decorated filaments. The low viscosity of solutions of ADF-F-actin is also consistent with a decreased rigidity due to the destabilization of longitudinal bonds.

Similar conclusions can be derived from the analysis of the kinetics of ADF binding to F-actin maintained in different structural states by phalloidin, by proteolytic modification, or by capping by gelsolin. The highly ordered structure of the
filament stabilized by phalloidin or P/BeF$_3$ is not favorable for ADF binding. In contrast, ADF binds faster and in a non-cooperative fashion to gelsolin-capped filaments or to filaments assembled from subtilisin-cleaved actin, which both display a bridge of density connecting the two strands of the long pitch helix (27), which suggests that the structure of the filaments in which lateral bonds are strengthened is favorable for ADF binding. If ADF were binding preferentially to bent regions of filaments, as proposed to account for its putative severing function (11), opposite results would have been obtained, i.e. ADF would have bound less easily to short rod-like gelsolin-capped filaments and to subtilisin-cleaved F-actin, which has a large persistence length (30). The cooperative change in structure of the filament linked to ADF binding, accompanied by a torsional movement (14) and a possible decrease in length, is expected to cause a large mechanical constraint if the filament is immobilized between two anchorage points. This constraint may result in breakage of the filament, which has in fact been observed in video microscopy experiments when filaments were immobilized on myosin heads in rigor prior to addition of ADF (11). In conclusion, our work brings more support to the view that the variability of the F-actin structure can be modulated by actin-binding proteins.

The fact that ADF binds faster to gelsolin-capped filaments may be physiologically relevant in actin-based motility, as follows. The forward movement of the leading edge is thought to be due to the steady-state barbed end growth of non-capped filaments at the membrane, whereas filaments that dissociate from the membrane become capped and undergo depolymerization from their pointed ends at the rear of the lamellipodium (15). The present results suggest that ADF may enhance the treadmill process supporting protrusion of the lamellipodium by binding selectively to the capped filaments and activating their depolymerization, whereas the uncapped filaments at the front would be less accessible to ADF and therefore would keep a stiffer structure appropriate for pseudodop extension. The presence of rigid stretches of F-actin ADP-P$_i$ subunits (30) at the growing barbed ends, which remain ADF-free, would further enhance the functional sorting of filaments. Further experiments will have to be designed to test the possibility that, in modulating the structure of the filaments, ADF may as well generate spatial order in the cell, e.g. in selecting different types of myosin motors to translocate particles to and from different subcellular locations.

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