Analogous F-actin Binding by Cofilin and Gelsolin Segment 2 Substantiates Their Structural Relationship*

(Received for publication, August 20, 1997, and in revised form, October 14, 1997)

Marleen Van Troyes, Daisy Dewitte, Jean-Luc Verschelde, Mark Goethals, Joël Vandekerckhove, and Christophe Ampe‡

From the Flanders Interuniversity Institute for Biotechnology, Department of Biochemistry, Faculty of Medicine, Universiteit Gent, Ledeganckstraat 35, 9000 Gent, Belgium

Cofilin is representative for a family of low molecular weight actin filament binding and depolymerizing proteins. Recently the three-dimensional structure of yeast cofilin and of the cofilin homologs destrin and actophorin were resolved, and a striking similarity to segments of gelsolin and related proteins was observed (Hatanaka, H., Ogura, K., Moriyama, K., Ichikawa, S., Yahara, I., and Inagaki, F. (1996) Cell 85, 1047–1055; Fedorov, A. A., Lappalainen, P., Fedorov, E. V., Drubin, D. G., and Almo, S. C. (1997) Nat. Struct. Biol. 4, 366–369; Leonard, S. A., Gittis, A. G., Petrella, E. C., Pollard, T. D., and Lattman, E. E. (1997) Nat. Struct. Biol. 4, 369–373). Using peptide mimetics, we show that the actin binding site stretches over the entire cofilin α-helix 112–128. In addition, we demonstrate that cofilin and its actin binding peptide compete with gelsolin segments 2–3 for binding to actin filaments. Based on these competition data, we propose that cofilin and segment 2 of gelsolin use a common structural topology to bind to actin and probably share a similar target site on the filament. This adds a functional dimension to their reported structural homology, and this F-actin binding mode provides a basis to further enlighten the effect of members of the cofilin family on actin filament dynamics.

Cofilin belongs to a family of actin modulating proteins that is widely distributed throughout eukaryotes. Homologs have been identified in vertebrates, plants, yeast, Drosophila, Caenorhabditis, Acanthamoeba, and Dictyostelium (Ref. 1 and references therein). In vivo they are found in the highly motile periphery of the cell (2, 3), and overexpressing cofilin in Dictyostelium cells stimulates their motility (4). Cofilin appears essential for yeast growth (5, 6) and is important during cytokinesis in Drosophila (7) and Xenopus (8).

Cofilin family members are multifunctional in vitro, displaying binding to both monomeric and filamentous actin. Cofilin and actin depolymerizing factor (ADF) or destrin are functionally equivalent, and the mechanism by which they enhance filament turnover has recently been addressed (9–11). In an important study, Carlier et al. (9) provide evidence that members of the cofilin family act by accelerating the rate of treadmilling because they increase the rate of dissociation from the pointed end of the filament and consequently, by providing a large monomer pool, also the rate of association to the barbed end. Consistent with this notion is the observation that the rate of propulsion and/or the length of the tails of locomoting Listeria (i.e. the rate of turnover of filaments in the tail) is strongly dependent upon ADF (9, 12, 13). Carlier et al. (9) show that in vitro this activity results in a new steady state with a larger monomer pool (of G-actin and G-actin-ADF) that varies with pH. This explains earlier observations that show that these proteins mainly bind to the actin filament in a 1:1 molar ratio to actin protromers at near neutral pH, but at slightly more alkaline pH these proteins induce actin depolymerization to a larger extent (14, 15).

The interaction of cofilin and ADF with actin is inhibited by phospholipids and regulated by phosphorylation, making them prime candidates as stimulus responsive mediators of actin dynamics (16, 17). Phosphorylated ADF and cofilin are unable to bind to actin, and in vivo dephosphorylation of these proteins is correlated with cytoskeletal reorganization (18, 19).

A region in the COOH-terminal half of cofilin has been demonstrated to participate in actin binding. By peptide mapping of a cross-linked actin-cofilin complex and mutational analysis of cofilin, lysine residues 112 and 114 were shown to interact with actin (20). These residues are part of the motif 111LKSKM115 that shows homology with actin binding motifs in other actin modulating proteins (21). A synthetic peptide patterned around these residues (Trp104-Met115) inhibits actin polymerization and induces depolymerization (22). However, the sequence formed by cofilin residues Asp122-Leu129 (homologous to the NH2 terminus of tropomyosin) has also been implicated in actin binding as the corresponding synthetic heptapeptide competes with cofilin for binding to actin (23). Both amino acid sequences Trp104-Met115 and Asp122-Leu129 are highly conserved throughout the cofilin/ADF family.

We hypothesized that these two neighboring motifs, which in previous studies were considered as separate actin binding entities (22, 23), essentially are part of the same site. Therefore, we studied the properties of a chemically synthesized peptide that spans both sites of interest, and we show that both motifs cooperate and actually form one actin binding unit. These data are supported by the recently determined, three-dimensional structure of cofilin (24). Specifically, the two neighboring motifs Trp104-Met115 and Asp122-Leu129 (homologous to tropomyosin) are correlated with cytoskeletal reorganization (18, 19).

This paper is available on line at http://www.jbc.org

* This work was supported by the Human Capital and Mobility Program of the European Community CHRX 0430, by Grant GOA-91/96-3 and Grant G.0060.96 of the Belgian National Science Foundation (to C. A.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Research associate of the F.W.O. Vlaanderen. To whom correspondence should be addressed: Dept. of Biochemistry, Faculty of Medicine, Universiteit Gent, Ledeganckstraat 35, B-9000 Gent, Belgium. Tel.: 32 9 264 53 06; Fax: 32 9 264 53 37; E-mail: Champ@gengenp.rug.ac.be.

1 The abbreviations used are: ADF, actin depolymerizing factor; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; P3, cofilin actin binding peptide (residues 102–130); PS1, gelsolin segment 1 actin binding peptide (residues 88–117); PS2, gelsolin segment 2 actin binding peptide (residues 198–227); S1, gelsolin segment 1; S2, gelsolin segment 2; S2–3, gelsolin segment 2 to 3; TFE, 3,3,3-trifluoroethanol; PAGE, polyacrylamide gel electrophoresis; Pipes, 1,4-piperazinediethanesulfonic acid.
dimensional structures of yeast cofilin and the cofilin homologs destrin and actophorin which all have a fold similar to segments of proteins of the gelsolin family (24–26). Based on the, albeit weak, sequence similarity between cofilin and gelsolin segment 2 (S2), we performed experiments to show that both the actin binding peptide of cofilin and the intact protein compete for binding to F-actin with the filament binding domain of gelsolin (segment 2–3). Our competition data strongly suggest that the cofilin family and gelsolin S2 use a common structural topology to bind the actin filament and interact with a similar target site. At least in their F-actin binding properties, proteins of the cofilin family may thus be considered as functional homologs of segment 2 of gelsolin that have evolved to be differently regulated.

EXPERIMENTAL PROCEDURES

Proteins and Peptides

We prepared rabbit skeletal muscle actin following the procedure of Spudich and Watt (27) and isolated it as CaCl\textsubscript{2}–ATP–G-actin by chromatography over Sephadex G-200 in G buffer (5 mM Tris–HCl, pH 7.7, 0.1 mM CaCl\textsubscript{2}, 0.2 mM ATP, 0.2 mM dithiothreitol, 0.1% NaN\textsubscript{3}). Actin was labeled with N-4-pyrenyldodecaneamide as described (28). We prepared human plasma gelsolin, digested it with α-chymotrypsin, and purified the obtained gelsolin fragments following the procedure of Bryan (29). Cofilin was purified from porcine brain based on the protocol of Yonawara et al. (30) with slight modifications. We used butyl-Sepharose as hydrophobic interaction column, followed by a hydroxypatite column. This yielded a cofilin preparation only contaminated with a 25-kDa protein (M\textsubscript{app}) which we identified as phosphatidylyethanolamine-binding protein. Attempts to purify cofilin further systematically resulted in the loss of the protein. We used the mixture whereby phosphatidylyethanolamine-binding protein serves as an internal control since it does not interact with G- or F-actin.

Cofilin peptides and the gelsolin segment 2 and segment 1 peptides were chemically synthesized on a model 431A peptide synthesizer (Apotronics since it does not interact with G- or F-actin.

Circular Dichroism Measurements

We performed measurements on a Jasco J-710 spectropolarimeter scanning from 184 to 200 nm with a step resolution of 0.5 nm. The samples, at a concentration of 40 μM, were in 10 mM sodium phosphate, pH 7.5, and 3,3,3-trifluoroethanol (TFE, 60%) and at 20 °C. The data are the average of nine scans and are expressed as δ\textsubscript{cw} (mean residue ellipticity) as a function of wavelength.

Competition Experiments

Sedimentation Assay—G-actin (initial concentration >12 μM) was allowed to polymerize in 75 mM Pipes, pH 6.9, 0.02 M dithiothreitol, 0.2 mM ATP, 0.1 mM CaCl\textsubscript{2} containing 100 mM KCl and 1 mM MgCl\textsubscript{2}. The final actin concentration after addition of all components will be 5 μM. Next we added S2–3 and cofilin (in the same buffer as actin) in such a way that the sum of both was 10 μM throughout the whole series, but their molar ratio varied, with 10:0 and 0:10 forming the two extremes (see Fig. 4, a and b). This 2-fold molar excess over actin is, for both S2–3 and cofilin separately, sufficient to saturate F-actin. The samples were kept at 4 °C overnight after a 30-min incubation at room temperature. F-actin and its associated proteins were subsequently spun down in an Beckman Airfuge at 30 p.s.i. (100,000 × g) for 30 min at room temperature. Supernatant and resuspended pellet were analyzed on SDS-mini slab gels followed by Coomassie Blue staining. We used densitometry to quantify the results.

From the equilibrium dissociation constant of the actin-cofilin (K\textsubscript{c}) and actin-S2–3 complex (K\textsubscript{g}) we can derive equations (Equations 1 and 2) that render a relation between either the amount of actin-cofilin (AC) or actin-S2–3 (AS) formed as a function of the total concentration of cofilin (C\textsubscript{a}).

\[
C_{\text{ac}} = \frac{(5 + 5n)(AC) + (1 - n)(AC)^2}{5n - (n - 1)(AC)} \quad (\text{Eq. 1})
\]

\[
C_{\text{ag}} = \frac{(1 - n)(AS)^2 + (5n - 15)(AS) + 50}{5 + (n - 1)(AS)} \quad (\text{Eq. 2})
\]

This takes into account that the sum of the total added concentration of cofilin and S2–3 is always 10 μM, and we assume that the sum of the amount of actin-cofilin (AC) and actin-S2–3 (AS) complex is constant in all samples as the filament is saturated (at two out of 12 data points (C\textsubscript{ac} = 6 and 7 μM) this may not be the case). This sum equals 5 μM (this is not taking into account the critical monomer concentration for actin polymerization and the limited extent of depolymerization induced by cofilin at pH 6.8 (which maximally amounts to about 25%). n is the ratio of the two equilibrium dissociation constants K\textsubscript{c}/K\textsubscript{g}. Using these equations, curves can be drawn for all possible n values, and the one fitting our experimental data has a n value between 2.5 and 3, which implicates that under our conditions (and with the introduced assumptions) K\textsubscript{g} is estimated to be 2.5–3 fold K\textsubscript{c}, which is in the range of reported K\textsubscript{g} values for binding of actin to S2–3 (ranging from 0.2–2 μM for S2–3 (F-actin)) and to cofilin (0.2–0.9 μM (G-actin)) (34–36).

Cross-linking—We added the first actin-binding protein or peptide in a constant amount to several samples of polymerized actin in phosphate buffer (see above) and incubated them for 1 h at room temperature. At this time the “competitive” peptide or protein was added in increasing concentrations to the different samples. After incubation to allow complex formation (overnight at 4 °C), we added the cross-linker EDC and N-hydroxysulfosuccinimide and let them react for another 45 min at room temperature. SDS gels were run for analysis.

To assay the competition between PS2 and P3 for cross-linking to F-actin, we used PS2 labeled with \textsuperscript{14}C on the NH\textsubscript{2}-terminal cysteine. PS2 was added in a range of 3–250 μM to 12 μM polymerized actin preincubated with either 150 μM P3 or without P3. Competition between PS2 and P3 was studied in the same way. Conversely, F-actin (12 μM) was first incubated with labeled PS2 (using either 20, 40, or 100 μM), and subsequently, P3 was added (range 0–200 μM). In both setups, the yield of labeled PS2 cross-linking to actin was quantified using a PhosphorImager (Molecular Dynamics) and the ImageQuant software package.

Immunoassays

The rabbit polyclonal anti-actin antibody against the COOH terminus was produced by the Centre d’Economie rurale, Laboratoire d’Hormonologie (Marloie, Belgium). A chemically synthesized actin peptide (residues 354–375) coupled to keyhole limpet hemocyanin fol-
RESULTS

Activity of the Wild Type Cofilin Peptide on Actin—We analyzed the actin binding capacity of a peptide that combines two regions of cofilin which have previously been implicated in actin binding. This peptide (P3, Fig. 1) spans residues 102–130 of the pig cofilin sequence and contains the LKSKM motif (studied in Ref. 22) and the more COOH-terminally located DAIKKKKL motif (studied in Ref. 23). The shorter peptides P1 (residues 104–115) and P2 (residues 118–130) each containing one motif served as control (Fig. 1).

Using a similar assay as described in Ref. 22, we first examined the inhibitory effect of these peptides on salt-induced actin polymerization by following the fluorescence increase accompanying the polymerization of pyrene-labeled actin (10% labeled). Although intact cofilin appeared to quench fluorescence of the pyrene label (39), we and others (22) did not observe this for the actin binding peptide. Fig. 2a shows that P3 slows down actin polymerization in a concentration-dependent fashion as was also shown for P1 (22). However, peptide P3 is more active compared with the control peptides P1 and P2, indicating that this long peptide binds actin monomers more efficiently. This indicates that both motifs cooperate in the actin interaction.

Adding a 50-fold molar excess of P3 (i.e. 600 μM) over actin at the start of polymerization results in only 14.5 and 65.5% of the F-actin formed at the half-time and at the time of complete polymerization in the control sample, respectively (Fig. 2a). The control curve (thick line) shows the polymerization of 12 μM actin (10% pyrenylated) induced by addition of salts at time 0. The cofilin wild type peptides are added at the indicated molar excess over actin at time 0 together with the salts (P1, circles; P2, triangles; P3, squares with open and closed symbols for 25- and 50-fold molar excess, respectively). The dashed lines indicate the time points of complete (100%) and 50% polymerization in the control sample and serve to facilitate comparison of the inhibitory effect. b, chemical cross-linking of the wild type cofilin peptide P3 to F-actin. The amount of chemical cross-link formed by wild type cofilin peptide (P3, 14C-labeled on methionine 115) to F-actin at an actin concentration of 12 μM as a function of P3 concentration (indicated above each lane in μM) was analyzed on an SDS gel, followed by autoradiography (see inset). The band of the actin-P3 (14C) cross-link appears double on the autoradiogram, likely due to the presence of more than one cross-linking event per actin-P3 complex which may give rise to multiple bands on the gel. The graph shows the degree of P3 cross-linking to actin as a function of P3 concentration based on a quantification using a PhosphorImager.

From the three-dimensional structure of the mammalian cofilin homolog destrin (24) and of yeast cofilin (25), it is evident that the sequence corresponding to P3 contains a kinked α-helix (residues 112–128). Consequently, when the peptide binds to actin it must become α-helical. Using circular dichroism, we show that P3 is capable of adopting an α-helical conformation (Fig. 3). This structure is not stable in aqueous solution but becomes evident upon addition of 60% 3,3,3-trifluoroethanol (TFE), a known α-helix stabilizing agent (40). In contrast, P1 does not adopt an α-helical conformation under these conditions, and the shorter peptide P2 is also less helical than P3.

Common F-actin Binding Properties between Cofilin and the F-actin Binding Segment of the Gelsolin Family—The structural homology between destrin and segments of gelsolin, as
Analogous F-actin Binding by Cofilin and Gelsolin

for binding to F-actin using a continuous variation experiment. Fig. 4b shows the composition of the pellets after high speed centrifugation of a series of samples in which the molar ratio of S2–3 and cofilin varies, but their total molarity (S2–3 + cofilin) remains constant at a 2-fold molar excess over actin. The amount of each complex formed (either actin-cofilin or actin-S2–3) is determined from the amount of cofilin and S2–3 in the pellet after high speed centrifugation and plotted in Fig. 4a (solid lines) as a function of total cofilin concentration. The fact that, in the presence of cofilin, the amount of S2–3 bound to F-actin is lower, compared with samples containing equal concentrations of S2–3 but no cofilin (and vice versa), and that the reduction in the F-actin bound S2–3 is compensated by an increase in bound cofilin of about similar size indicates that cofilin and S2–3 displace each other and therefore suggests that both proteins compete for the same or an overlapping site on F-actin.

In addition, densitometric scanning of cross-linking experiments shows that a saturable amount of the actin-binding peptide of cofilin (250 μM P3) decreases the yield of cofilin binding to F-actin (Fig. 4c). The same effect was observed for the binding of cofilin in the presence of the segment 2 gelsolin peptide PS2. This chemically synthesized peptide contains part of the F-actin binding site of gelsolin segment 2 (residues 198–227, see Fig. 1), competes with S2–3 for binding to F-actin, and slows down actin filament severing by gelsolin (44). The actin binding peptide from gelsolin segment 1 PS1, shown by Feinberg et al. (45) to also interact with F-actin, has only a small or no effect on the yield of cross-linking of cofilin. In a converse experiment, P3 reduces the yield of S2–3 cross-linking to F-actin (Fig. 4d).

Direct competition between the cofilin P3 and gelsolin PS2 peptides assayed by chemical cross-linking cannot be visualized on SDS gels using Coomassie staining due to the nearly identical molecular weight of both actin-peptide cross-links. We therefore labeled the cysteine residue at the NH2 terminus of PS2 with 14C. Fig. 4e shows that the yield of cross-linking of PS2 to F-actin is reduced over a large concentration range of PS2 when the F-actin is preincubated with 150 μM P3, whereas it is not hindered by a preincubation with PS1 (150 μM). Fig. 4f shows the results of three separate experiments in which we preincubated F-actin with three different concentrations of labeled PS2, subsequently added P3 in increasing concentrations, and looked at the effect on the amount of actin-PS2 cross-link formed. At each of the three PS2 concentrations tested, an increasing concentration of P3 reduces the amount of actin-PS2 cross-link.

Taken together, these data strongly suggest that members of the cofilin family bind to the same or to an overlapping site on F-actin as segment 2 of gelsolin. The fact that (i) S2–3 and the small (29-mer) cofilin-actin binding peptide, (ii) cofilin and the gelsolin F-actin binding peptide, and (iii) also the two actin binding peptides mutually compete reduces the chance that competition is caused by nonspecific steric hindrance, whereas the assumption that both interact with the same site on F-actin becomes more likely.

Study of the Cofilin Actin Target Site Using Site-specific Anti-actin Antibodies—Hatanaka et al. (24) modelled destrin on the gelsolin segment 1 actin binding site, i.e. in the cleft between actin subdomain 1 and 3. They present this as a plausible binding mode despite the differences in charge distribution at the site of actin binding (also observed in Ref. 26) which they explain in terms of a different Ca2+ regulation. In an attempt to obtain information about the cofilin binding site in actin and/or to possibly discriminate between the site proposed in Ref. 24 and another one, we screened the cross-linked
Fig. 4. a, competition between cofilin and S2–3 for binding to F-actin. Quantification (using densitometry) of SDS-PAGE analysis of the sedimentation of F-actin with either S2–3 (▲) and cofilin (×) alone (dashed lines) or in a continuous variation experiment in which the sum of S2–3

Analogous F-actin Binding by Cofilin and Gelsolin
plexes of actin-P3 or actin-cofilin for recognition by antibodies raised against specific parts of the actin molecule. If the antibody fails to recognize the cross-linked complex, this suggests that the cross-linked partner blocks the epitope on actin (see e.g. Ref. 44). We used an antibody that specifically recognizes actin residues 354–375 (the extreme COOH terminus and consequently part of the gelsolin S1 target site). A second (commercial) antibody is specific for α-sarcomeric actin, and we determined its epitope lies between residues 12 and 44 (part of subdomain 1 and 2). The actin-cofilin peptide P3 cross-linked complex and the actin-cofilin complex are still recognized by the anti-COOH terminus and by the anti-α-sarcomeric actin antibody (Fig. 5, a and b).

**DISCUSSION**

We provide insight into the actin filament binding properties of cofilin, a representative of the family of small actin filament modulating proteins, in light of the recently determined three-dimensional structure of destrin (24) and yeast cofilin (25). We also studied the relation of cofilin to gelsolin which allows us to hypothesize on the binding site of cofilin on F-actin.

We show that the cofilin peptide P3 (residues 102–130) containing both actin binding motifs, previously considered as separate actin binding peptides (22, 23), is more effective in interacting with actin than the respective short peptides containing only one motif (P1 and P2). The use of small peptides to mimic the interaction with actin has proven to be a valid approach (46), although they cannot be expected to contain all binding information; one needs the intact protein to have a fully active entity. Lappalainen et al. (47) recently demonstrated that also other cofilin regions are involved in the F-actin interaction. This may explain the difference in the affinity and activity of the cofilin peptide P3 versus total cofilin. The peptide just slows down polymerization, whereas the total protein induces an overshoot during polymerization. However, the fact that saturation of binding is reached with P3, combined with the observation that this peptide competes with intact cofilin for binding to actin filaments, proves the specificity of binding in a manner similar to that of the intact protein.

The three-dimensional structures of destrin (24) and actophorin (26), two cofilin homologs (71 and 40% identity, respectively (48, 49)) and of yeast cofilin (25) reveal that the sequence of P3, used here, spans part of a β-strand (residues 102–106), a loop region, and a long α-helix (residues 112–128). This α-helix, in which the two basic actin binding motifs are located at both ends, belongs to the most conserved region in this family of proteins. Although P3 residues that correspond to the β-strand and turn are not expected to fold correctly in the absence of the rest of the β-sheet, P3 can form the α-helix, which is markedly more stable than the ones formed by the shorter peptides P1 and P2 as judged by our CD data. Consequently, the higher activity of P3 results from the combinatorial effect of containing the two actin interacting motifs as well as from the higher propensity to form the “active” α-helical configuration. Taken together, our peptide mimetics show that the entire α-helix participates in cofilin binding to F-actin. This is further supported by our findings, based either on cofilin peptide mimetics or on site-directed mutagenesis of intact actophorin, that changing the basic residues in both motifs reduces binding activity.

Proteins of the cofilin family are able to interact with filamentous actin, and consequently their behavior in relation to other F-actin binders is of prime interest, the more so as the cofilin fold (24, 25, 26) is very similar to that of the repeated segments of gelsolin (50). This suggested to us that gelsolin and cofilin share some functional similarities, and the biochemical data we present here support this idea. However, in addition, they allow us to postulate that cofilin is a gelsolin segment 2 homolog with regard to F-actin binding. We opted to use the proteolytically derived S2–3 fragment, known to be in an active conformation, S2 and S2–3 bind to the actin filament with nearly the same affinity (34, 51), whereas isolated S3 hardly interacts with F-actin (34), strongly indicating that S2 contains the F-actin binding site that resides in S2–3. The recently resolved structure of total, Ca^{2+}-free gelsolin (50) shows that S2 and S3 are connected with a 23-amino acid long linker and that S3 folds back on S1 in the S1–S3 triplet. As S3 contains no actin binding site (34), this segment may mainly function to stabilize the relative orientation between the actin binding segments S1 and S2 that will change upon Ca^{2+} activation.

In the absence of biochemical data, Hatanaka et al. (24) modelled destrin in an S1-like binding mode on actin (in the cleft between actin subdomain 1 and 3 (52)); however, our data make it more plausible that cofilin family members bind to the gelsolin S2 target site of F-actin, which is proposed to lie between the subdomain 1 of two adjacent protomers along the axis of the actin filament (53) (see also below). Indeed, we show...
that cofilin competes with the F-actin binding fragment of human plasma gelsolin (segment 2–3 fragment) for binding to polymerized actin. The continuous variation experiment shows that, at a S2–3 concentration that allows saturation of the filament, the binding of cofilin to an actin protomer implies the dissociation of S2–3 and vice versa. A competitive behavior of this kind evokes two possible mechanisms. Either the two proteins bind to a same or a similar target site (here along the filament), in which competition means a true displacement, or they bind to a different site (here for instance the gelsolin S1 target site) with an accompanying induced conformational change in actin or steric hindrance leading to mutually exclusive binding. Although we cannot completely exclude the latter option, several data taken from the literature and in particular those presented here argue that the cofilin and S2–3 binding sites on actin are at least similar and differ from the S1 binding site.

The first argument for a similar target site of cofilin and S2 lies in the fact that competition is maintained between each protein and the actin binding peptide of the other protein, i.e. between cofilin and the gelsolin S2 F-actin binding peptide PS2 and between gelsolin fragment S2–3 and the cofilin actin binding peptide P3. More importantly, the two actin binding peptides compete themselves. This strongly suggests these proteins have a similar target site on the filament, especially in view of the small size of the peptides, an alternative explanation based on steric hindrance, for example, becomes unlikely. In addition, we show that the gelsolin S1 peptide PS1 does not hinder binding of cofilin or PS2 to F-actin; this also argues against the model proposed by Hatanaka et al. (24).

Structural data on cofilin family members (24–26) and on total gelsolin (50) show that the sequences of P3 and PS2 span structurally analogous α-helices in the cofilin/destrin and gelsolin segment 2 structures, although they appear different in sequence. Different sequences binding to the same target site on actin is not unprecedented in actin binding proteins. A striking example is discussed in a recent report comparing profilin residues implicated in actin binding based on the plant, Acanthamoeba and bovine profilin crystal structures (the proteins themselves have very little sequence conservation). Only 4 out of 15 residues involved in actin binding are identical (see Table III in Ref. 54). Moreover, an examination of the sequences of P3 and PS2 show they may not be that different. Indeed, the basic residues Lys114 and Lys125 in the cofilin α-helix have been implicated in actin binding (Lys114 (22, 47), Lys114 and Lys125, Footnote 2) and are strongly conserved in the cofilin family. The analogous residues in segment 2 of gelsolin and of all other members of the gelsolin family are also basic in nature, except for the analogous residue of Lys225 in the lower eukaryote form severin which is an aspartate. Interestingly, in contrast to gelsolin, actin binding by severin segment 2 is Ca2+-dependent, suggesting a possible role for the charge difference. We believe that the conservation of these basic charges may indicate the importance of these residues for interacting with the same actin target site, the more so because in gelsolin segment 1 the nature of these residues is acidic or neutral. More generally, in contrast to the actin binding helices of cofilin and gelsolin S2, the actin binding helix of S1 does not contain positively charged residues. This is illustrated in Fig. 6 which shows a helical wheel presentation of the actin binding helices of cofilin, gelsolin segment 2, villin segment 2, and gelsolin segment 1.

Our immunodata also demonstrate a pronounced difference between the actin target site of profilin on the one hand and cofilin (this study) and gelsolin S2–3 (44) on the other. Acanthamoeba profilin is known to cross-link actin residue 364 in the extreme COOH terminus (55) and binds actin in the cleft between subdomain 1 and 3 similar to gelsolin segment 1 (56). Thus, our data argue against a profilin-like or gelsolin S1-like binding model for cofilin (and related proteins).

Taken together, the above data allow us to present a binding mode for cofilin in which it contacts actin via a similar target site as gelsolin S2. This site in actin has not yet been determined in detail, but using electron cryomicroscopy and helical reconstruction on complexes of F-actin with the F-actin binding segment of α-actinin (α-A1–2), which can functionally replace S2–3 in gelsolin segment 1–3 (34), McGough and co-workers

![Figure 6](image1.png)

**Fig. 6.** Helical wheel presentation of the actin binding helices of cofilin, gelsolin S1 and S2, and villin S2. The side of the helices oriented toward the core of the actin binding protein is indicated (dashed line). For gelsolin segment 1, the side that is interacting with actin is also indicated (dashed line with arrows) (52). The two basic clusters at the two sides of the cofilin helix are formed by the lysine residues that are shaded. The two basic residues that are conserved between cofilin and S2 of gelsolin and villin are indicated by arrows, as well as their counterparts in gelsolin S1 (lower left).

![Figure 7](image2.png)

**Fig. 7.** Model showing the proposed binding mode of cofilin to the actin filament in analogy with the binding mode of gelsolin segment 2 as presented in Refs. 53 and 57. In this model cofilin contacts two actin protomers. The position of the NH2 termini of the protomers is indicated as well as the subdomain numbers. For comparison the actin binding site of gelsolin segment 1 at the barbed end of the filament is also shown (52).
(53, 57) proposed that S2–3 is positioned along the filament and in contact with two longitudinally associated protomers. In this model, it contacts the outer face of subdomain 2 and subdomains 1 of the same and of the next protomer (Fig. 7). This confirms with earlier cross-linking data of actin and α-actinin (58). Very recently, these authors used the same techniques to study F-actin decorated with coflin and suggested a similar contact region on the filament (59). As these reconstructions are performed at a resolution of 25–30 Å, and in the case of coflin were additionally hampered by the induced conformational change in the filament, our data provide important biochemical support to this model. Moreover, we show that a structurally analogous α-helix of coflin and S2 is involved in the interaction.

However, although the dimensions of coflin can accommodate a bridging between adjacent subunits in the filament (24), the helix alone will not suffice to generate the large coflin (or S2)–actin interface proposed by McGough and co-workers (53, 59). Consequently, other regions of these proteins must be involved. For yeast coflin, this was recently demonstrated in an extensive mutagenesis study (47) which shows that F-actin (but not G-actin) binding also implies the participation of residues in the β-strand preceding the helix studied here, while also the extreme coflin NH2 and COOH termini are important. Similarly residues 150–172 (51, 60) in the NH2-terminal part of gelsolin segment 2 have also been implicated in actin binding. The structure of gelsolin (50) shows this region constitutes a single, solvent-exposed entity with the S2 actin binding helix 208–223.

Earlier biochemical observations also fit our model which places coflin on the gelsolin S2-binding site. First, coflin binds the filament in a 1:1 stoichiometry to protomers (9, 36) and, based on the combination of biochemical experiments and real time observation of the activity of actophorin (the coflin homolog from Acanthamoeba), Maciver et al. (43) proposed that actophorin binds along the filament. Second, coflin Lys312 or Lys114 were demonstrated to form an EDC cross-link with actin residues 1–12 (61). This cross-link between the NH2 terminus of the coflin actin binding helix and the extreme actin NH2 terminus again supports an S2-binding mode. Although not exactly in the proposed binding site, the NH2 terminus of either contacted protomer may be in close enough proximity to cross-link the actin binding helix of coflin (Fig. 7), the more so as the actin NH2 terminus displays a high flexibility (62). Third, an S2-binding mode for coflin can also explain the effect of coflin on nucleotide exchange (63) and the preference of members of the coflin/ADF family for ADP–versus ATP-actin (9, 64) as the conformation of subdomain 2, which is then assumed to be part of the binding site, is closely connected to the shape of the nucleotide cleft (65).

In conclusion, we propose that proteins of the coflin/ADF and gelsolin family interact with a similar target site on F-actin via a structurally analogous part. This concerns the initial way of contacting the filament and does not imply a subsequent similar effect on overall actin dynamics. It is evident that for both proteins families, binding to F-actin is differently regulated. In vitro activity of coflin is shown to vary with pH. As proposed by Hatanaka et al. (24), this pH dependence most likely involves histidine residues in actin. His30, His87, and His88 are located in actin subdomain 2 and in a loop connecting subdomains 1 and 2, i.e. in or near the proposed binding site for coflin. A change in any of these histidines may exert an effect on coflin bound to the filament.

Next to being pH-dependent, the coflin-actin interaction is also regulated by phosphorylation (19) and, in contrast to the activity of gelsolin, not dependent on Ca2+. F-actin binding by gelsolin S2 itself does not require Ca2+, but the Ca2+ dependence is imposed by the other segments of this multidomain protein. This different regulation of two actin binding proteins that are analogous in their initial interaction with the filament and have been localized to the same cellular regions provides the possibility to have a fine-tuned differential regulation of their mutual activity in vivo. In this respect, the recently presented “ADF-enhanced funnelled treadmilling process” by Carlier and Pantaloni (66) demonstrates how the interplay of proteins of the coflin family with capping proteins can strongly potentiate actin dynamics.

Acknowledgments—We thank Prof. Dr. M. Rosseneu for use of the spectropolarimeter to perform the circular dichroism experiments, Prof. Dr. M. Van Montagu for use of the PhosphorImager, Prof. S. De Boeck for helpful discussion, and J. Van Damme for performing mass measurements.

REFERENCES

1. Moon, A., and Druhin, G. D. (1995) Mol. Biol. Cell 6, 1423–1431
2. Bamburg, J. R., and Bray, D. (1987) J. Cell. Biol. 105, 2817–2825
3. Aizawa, H., Sutoh, K., Tsushima, S., Kawashima, S., Ishii, A., and Yahara, I. (1985) J. Biochem. 97, 4021–4026
4. Aizawa, H., Sutoh, K., and Yahara, I. (1996) J. Cell. Biol. 132, 335–344
5. Iida, K., Moriyama, K., Matsumoto, S., Kawasaki, H., Nishida, E., and Yahara, I. (1993) Gene (Amst.) 124, 115–120
6. Moon, A., Janney, P. A., Louie, R. A., and Druhin, D. G. (1993) J. Cell Biol. 120, 421–435
7. Gunsalus, K. C., Bonaccorsi, S., Williams, E., Verni, F., Gatti, M., and Coldberg, M. L. (1995) J. Biol. Chem. 270, 1243–1259
8. Abe, H., Ohinata, T., Minamide, I., and Bamberg, J. R. (1996) J. Cell Biol. 132, 871–885
9. Carlier, M.-F., Santolini, J., Laurent, V., Didry, D., Yan, H., Chua, N.-H., and Pantaloni, D. (1996) Mol. Biol. Cell 7, 546
10. Weeds, A. G., Pope, B., Whyteck, S., and Maciver, S. (1996) Mol. Biol. Cell 7, 202a
11. Carlier, M.-F., Laurent, V., Santolini, J., Melki, R., Didry, D., Xia, G.-X., Hong, Y., Chua, N.-H., and Pantaloni, D. (1997) J. Cell Biol. 136, 1307–1323
12. Rosenblatt, J., Agnew, B. J., Bamburg, J. R., and Mitchison, T. J. (1996) Mol. Biol. Cell 7, 546a
13. Rosenblatt, J., Agnew, B. J., Abe, H., Bamburg, J. R., and Mitchison, T. J. (1997) J. Cell Biol. 136, 1323–1332
14. Yonezawa, N., Nishida, E., and Sakai, H. (1985) J. Biol. Chem. 260, 14410–14412
15. Hayden, S. M., Miller, P. S., Brauwweiler, A., and Bamburg, J. R. (1993) Biochemistry 32, 10004–10004
16. Yonezawa, N., Nishida, E., Iida, K., Yahara, I., and Sakai, H. (1990) J. Biol. Chem. 265, 8382–8386
17. Davidson, M. L., and Haslam, B. R. (1994) J. Biol. Chem. 270, 367–370
18. Yonezawa, N., Nishida, E., Iida, K., Yahara, I., and Sakai, H. (1991) J. Biol. Chem. 266, 7248–7254
19. Vanroys, M., Dewitte, D., Goethals, M., Carlier, M.-F., Vandekerckhove, J., and Ampe, C. (1996) J. Biol. Chem. 271, 235–238
20. Yonezawa, N., Nishida, E., Kuboyama, M., and Sakai, H. (1991) J. Biol. Chem. 266, 10485–10489
21. Yonezawa, N., Nishida, E., Kuboyama, M., and Sakai, H. (1989) Eur. J. Biochem. 183, 235–238
22. Hatanaka, H., Ogura, K., Moriyama, K., Ishikawa, S., Yahara, I., and Inagaka, F. (1996) Cell 85, 1047–1055
23. Fedorov, A. A., Lappalainen, P., Fedorov, E. V., Druhin, D. G., and Almo, S. C. (1997) Nat. Struct. Biol. 4, 366–369
24. Leonard, S. A., Gittis, A. G., Petrella, E. C., Pollard, T. D., and Lattman, E. E. (1983) Anal. Biochem. 137, 371–376
25. Staros, J. V., Wright, R. W., and Suringle, D. M. (1986) Anal. Biochem. 156, 220–222
26. Way, M., Pope, B., and Weeds, A. G. (1992) J. Biol. Chem. 269, 335–338
27. Yin, H. L., Iida, K., and Janney, P. A. (1988) J. Biol. Chem. 263, 805–812
28. Muneyuki, E., Nishida, E., Sutoh, K., and Sakai, H. (1985) J. Biol. Chem. (Tokyo) 260, 563–566
29. Munsky, M. B., and Glanzman, A. G. (1991) Methods Enzymol. 195, 215–253
30. Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4550–4554
31. Nishida, E., Maekawa, K., Muneyuki, E., and Sakai, H. (1984) J. Biochem. (Tokyo) 95, 387–398
32. Nelsen, J. W., and Kallenbach, N. R. (1986) Proteins Struct. Funct. Genet. 1, 211–217

32577
41. Weeds, A. G., and Maciver, S. (1993) *Curr. Opin. Cell Biol.* **11**, 835–842
42. Hawkins, M., Pope, B., Maciver, S. K., and Weeds, A. G. (1993) *Biochemistry* **32**, 9885–9993
43. Maciver, S. K., Zot, H. G., and Pollard, T. D. (1991) *J. Cell Biol.* **115**, 1611–1620
44. Van Troys, M., Dewitte, D., Goethals, M., Vandekerckhove, J., and Ampe, C. (1996) *FEBS Lett.* **397**, 191–196
45. Feinberg, J., Mery, J., Heitz, F., Benyamin, Y., and Roustan, C. (1997) *Biopolymers* **41**, 647–655
46. Friederich, E., Vancompernolle, K., Huet, C., Goethals, M., Finidori, J., Vandekerckhove, J., and Leuward, D. (1992) *Cell* **70**, 81–92
47. Lappalainen, P., Fedorov, E. V., Fedorov, A. A., Almo, S. C., and Druhin, D. G. (1997) *EMBO J.* **16**, 5520–5530
48. Moriyama, K., Nishida, E., Yonezawa, N., Sakai, H., Matsumoto, S., Iida, K., and Yahara, I. (1990) *J. Biol. Chem.* **265**, 5768–5773
49. Quirck, S., Maciver, S. K., Ampe, C., Doberstein, S., Kaiser, D., Vandamme, J., Vandekerckhove, J., and Pollard, T. D. (1993) *Biochemistry* **32**, 8525–8533
50. Burtauck, L. D., Koepf, E. K., Grimes, J., Jones, E. Y., Stuart, D. I., McLaughlin, P. J., and Robinson, R. C. (1997) *Cell* **90**, 661–670
51. Sun, H.-Q., Wooten, D. C., Janmey, P. A., and Yin, H. (1994) *J. Biol. Chem.* **269**, 9473–9479
52. McLaughlin, P. J., Gooch, J. T., Mannherz, H.-G., and Weeds, A. G. (1993) *Nature* **364**, 685–692
53. McGouch, A., Way, M., and DeRosier, D. (1994) *J. Cell Biol.* **126**, 433–443
54. Thorne, K. S., Christensen, H. E. M., Shigeta, R., Jr., Huddler, D., Jr., Shalaby, L., Lindberg, U., Chua, N.-H., and Schutt, C. E. (1997) *Structure* **5**, 19–32
55. Vandekerckhove, J., Kaiser, D. A., and Pollard, T. D. (1989) *J. Cell Biol.* **109**, 619–626
56. Schutt, C. E., Myslik, J., Rosycki, M. D., Gonesekere, N. C. W., and Lindberg, U. (1993) *Nature* **365**, 810–816
57. McGouch, A., and Way, M. (1995) *J. Struct Biol.* **115**, 144–150
58. Mimura, N., and Asano, A. (1987) *J. Biol. Chem.* **262**, 4717–4723
59. McGouch, A., Pope, B., Chai, W., and Weeds, A. (1997) *J. Cell Biol.* **138**, 771–781
60. Kwiatkowski, D. J., Janmey, P. A., and Yin, H. L. (1989) *J. Cell Biol.* **108**, 1717–1726
61. Sutoh, K., and Mabuchi, I. (1986) *Biochemistry* **25**, 6186–6192
62. Kabesh, W., and Vandekerckhove, J. (1992) *Annu. Rev. Biophys. Biomol. Struct.* **21**, 49–76
63. Nishida, E. (1985) *Biochemistry* **24**, 1160–1164
64. Maciver, S. K., and Weeds, A. G. (1994) *FEBS Lett.* **347**, 251–256
65. Muhlrad, A., Cheung, P., Phan, B., Miller, C., and Reisler, E. (1994) *J. Biol. Chem.* **269**, 11852–11858
66. Carlier, M. F., and Pantaloni, D. (1997) *J. Mol. Biol.* **269**, 459–467