Molecular Biology of the Cell
Vol. 6, 1423–1431, November 1995

Essay

The ADF/Cofilin Proteins: Stimulus-responsive Modulators of Actin Dynamics

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Monitoring Editor: Thomas D. Pollard

Whether a cell is harnessing actin polymerization to produce movement, preparing for cytokinesis, or assembling long-lived actin filament–based structures such as sarcomeres or microvilli, actin polymerization must be controlled in time and space. Changes in actin organization often occur on a time scale that dictates an intimate coupling of a signal transduction apparatus to the machinery that controls actin assembly. This is the case when a motile cell extends pseudopodia in the direction of a gradient of increasing chemoattractant. To mediate such responses, eukaryotic cells are equipped with a battery of actin-binding proteins (Stossel *et al.*, 1985; Pollard and Cooper, 1986). Several lines of evidence have now converged to implicate the ADF/cofilin proteins in particular as stimulus-responsive mediators of actin dynamics. The ADF/cofilins are endowed with multiple activities that can modulate assembly, and these activities are inhibited by a phosphorylation that occurs in vivo. Significantly, ADF/cofilins undergo rapid dephosphorylation when cells are exposed to stimuli that cause changes in cytoskeletal assembly. Additional regulatory possibilities are suggested by observations that the actions and activities of ADF/cofilins can be influenced by pH, PIP_2, the nature of the actin filament-bound nucleotide (ATP or ADP), inorganic phosphate, and possibly, translocation into the nucleus and tension on the actin filament.

THE ADF/COFILIN FAMILY OF PROTEINS

Actin monomers reversibly self-associate to form actin filaments (F-actin), the only form of actin known to be functional. The low molecular mass (15–22 kDa) ADF/cofilin proteins exhibit monomer-binding, filament-binding, filament-severing, and nucleotide dissociation–inhibiting activities in vitro, which suggest that the role of these proteins in the cell is to destabilize filaments and correspondingly increase the amount of monomeric actin. In this review, the extensive biochemical studies on the ADF/cofilin proteins are analyzed in the context of potential cellular functions and regulatory mechanisms.

Representatives of the ADF/cofilin family were independently identified in several organisms (see Table 1 for original sources and names). When sequence information was obtained, it became clear that all of these proteins are related (25–71% amino acid identity), and that ADF and destrin are the same protein (see Table 1 legend for references). The subsequent discovery of ADF/cofilin proteins in yeast (Iida *et al.*, 1993; Moon *et al.*, 1993), *Drosophila* (Edwards *et al.*, 1994), *Dictyostelium* (Aizawa *et al.*, 1995) and plants (Kim *et al.*, 1993) suggests that all eukaryotic cells have at least one ADF/cofilin protein. ADF/cofilins also share limited homology with coactosin (de Hostos *et al.*, 1993) and with the N-termini of drebrin (de Hostos *et al.*, 1993) and Abp1p (Moon *et al.*, 1993). Although coactosin, drebrin, and Abp1p bind to actin filaments, none appears to sever filaments (de Hostos *et al.*, 1993; Ishikawa *et al.*, 1994; Lee and Drubin, unpublished data), and coactosin does not bind to monomers (de Hostos *et al.*, 1993). Thus, coactosin, drebrin, and Abp1p should not be considered members of the ADF/cofilin family.

INTERACTIONS WITH MONOMERIC ACTIN

The concentration of unassembled actin in a cell is much higher than the critical concentration for assembly of pure actin, providing a large reserve of subunits that can be assembled in response to appropriate cues. Several types of monomer-binding proteins, including ADF/cofilin, profilin, thymosin β_4, and ASP-56/Srv2p may contribute to this unpolymerized pool (Gieselmann and Mann, 1992; Fechheimer and Zigmond, 1993; Nachmias, 1993; Sun *et al.*, 1995). Significantly, differences in the interactions of the monomer-binding proteins with actin and in their responses to regulatory signals suggest that there may be multiple pools of monomeric actin that can be regulated by distinct mechanisms. For example, monomer-binding proteins differ in how they affect and possibly, are affected by, which nucleotide (ATP or ADP) is bound to actin.

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Table 1. ADF/cofilin family of proteins

Protein[a]	Source	Name derivation	N-terminal sequence[b]	Potential actin contacts[b,c]
ADF	chicken	"actin-depolymerizing factor"	MASGVQVADE	WAPELAPLKSKMVYASSKDALRRAL
destrin	mammalian	"destroys filaments"	(same as ADF)	(same as ADF)
cofilin	mammalian	"cofilamentous protein"	MASGVAVSDG	WAPENAPLKSKMIYASSKDAIKKKL
depactin	starfish	"depolymerizes actin"	PQSGTAL-DE	WSMETANIKLKMKYSSTVGTLKSAT
actophorin	*Acanthamoeba*	"actin-carrying protein"	MSGIAVSDD	WAPDSAPIKSKMMYTSTKDSIKKKL
cofilin	*Saccharomyces*		MSRSGVAVADE	WSPDTAPVRSKMVYASSKDALRRAL
ADF	lily		MANSSSGMAVDDE	WSPDTSRVRSKMLYASTKDRFKREL

Filament-binding proteins exhibiting limited sequence similarity to ADF/cofilin proteins:

Protein	Original source	MW	Severs filaments	Binds to monomers	N-terminal sequence	Corresponding region (see above)
Abp1p	*Saccharomyces*	85 kDa	No	Unknown	MALEPIDYTTHSR	WCPDSAPLKTRASFAANFAAVANNL
coactosin	*Dictyostelium*	17 kDa	No	No	MADVSSTELKAAY	WCGEEVGPLAKANVSVHKASVKQVI
debrin	chicken	95/100 kDa	Unlikely[d]	Unknown	MAGVGFAAHRLEL	WVGEDVPDARKCACASHVAKIAEFF

[a] The first five entries are the originally-identified members of the ADF/cofilin family. The last two entries are yeast and plant homologues included for sequence comparisons.

[b] In sequence alignments, invariant residues are in bold.

[c] Regions implicated as actin contacts via crosslinking and peptide studies are underlined (see text).

[d] Severing by drebrins is unlikely because there was no apparent increase in unsedimented actin and electron microscopy showed long filaments (Ishikawa *et al.*, 1994). However, neither viscosity nor assembly experiments to definitively test for severing have been reported.

References: For original identification and naming of ADF (Bamburg *et al.*, 1980), destrin (Nishida *et al.*, 1984a), cofilin (Nishida *et al.*, 1984b), depactin (Mabuchi, 1983), and actophorin (Cooper *et al.*, 1986). For sequence report of ADF/destrin (Abe *et al.*, 1990; Adams *et al.*, 1990; Moriyama *et al.*, 1990b), murine cofilin (Moriyama *et al.*, 1990a), depactin (Takagi *et al.*, 1988), actophorin (Quirk *et al.*, 1993), yeast cofilin (Iida *et al.*, 1993, Moon *et al.*, 1993), lily ADF (Kim *et al.*, 1993), Abp1p (Drubin *et al.*, 1990), coactosin (de Hostos *et al.*, 1993), and drebrin (Kojima *et al.*, 1988). For recognition of homology to ADF/cofilin for Abp1p (Moon *et al.*, 1993), coactosin (de Hostos *et al.*, 1993), and drebrin (de Hostas *et al.*, 1993).

Profilin (Lal and Korn, 1985) and thymosin β_4 (Carlier *et al.*, 1993; Pantaloni and Carlier, 1993) preferentially bind to ATP·actin. One study determined that the affinity of ADF for ATP·actin is higher than for ADP·actin (K_D of 0.11 μM and 1.26 μM, respectively) (Hayden *et al.*, 1993). However, a different study (Maciver and Weeds, 1994) found that actophorin has a higher affinity for ADP·actin, and therefore might sequester actin into a different pool from actin sequestered by profilin and thymosin β-4. Why opposite conclusions were reached in the above two studies is presently not known but may have to do with how the experiments were done. S. Maciver (personal communication) now finds that ADF preferentially binds to ADP·actin, although the difference is not as great as for actophorin mainly because ADF binds ATP·actin tightly while actophorin binds weakly.

The monomer-binding proteins also have different effects on the rate of nucleotide exchange on the monomer: while ADF/cofilins (Nishida, 1985; Hawkins *et al.*, 1993; Hayden *et al.*, 1993) and thymosin β_4 (Goldschmidt-Clermont *et al.*, 1992) inhibit nucleotide exchange, profilin catalytically stimulates exchange (Mockrin and Korn, 1980). In principle, stimulation of nucleotide exchange on actophorin-ADP·actin complexes could disrupt the complex and release not only assembly-competent monomer, but also actophorin that is free to sever filaments. Profilin might catalyze this reaction (see unpublished observation in Hayden *et al.*, 1993) despite the fact that profilin and actophorin cannot be cross-linked to actin simultaneously (Maciver *et al.*, 1991b).

Phosphatidylinositol 4,5-*bis*phosphate (PIP_2) has been implicated in the regulation of several actin-binding proteins, including profilin (Lassing and Lindberg, 1985). As with profilin, the interaction of cofilin with actin is inhibited by PIP_2 and PIP, but cofilin is also inhibited (although less strongly) by PI (Yonezawa *et al.*, 1990). This suggests that lipids can differentially regulate distinct pools of actin monomers. However, a physiological role for such differential regulation has not been demonstrated. Co-injection of cofilin and PIP_2 reduces the effect of cofilin on actin reorganization (Nagaoka *et al.*, 1995b) but this does not demonstrate that this inhibitory mechanism normally operates in vivo. Moreover, studies by Yonezawa *et al.* (1990) demonstrate that PIP_2 inhibits DNase I nuclease activity and DNase I actin-binding activities, albeit more weakly than it inhibits ADF/cofilin. This is troubling as it challenges the specificity

of PIP_2 inhibition. Actophorin also binds to PIP_2 (Quirk *et al.*, 1993).

Another striking difference between profilin and ADF/cofilin is seen when comparing the rates of actin depolymerization that are induced by these two proteins. Depolymerization by profilin can be accounted for by a mechanism in which profilin sequesters actin monomers that are released from filament ends (Carlsson *et al.*, 1977). Depolymerization facilitated by the ADF/cofilin proteins is much more rapid, suggesting a mechanistic difference (see below) (Bamburg *et al.*, 1980; Nishida *et al.*, 1984a). Indeed, the names of most ADF/cofilin proteins stem from their ability to induce rapid filament depolymerization (see Table 1).

Although profilin, cofilin, and thymosin β_4 may each be involved in maintaining pools of actin that can be mobilized for polymerization (e.g., the classic profilactin/acrosomal process transition) (Tilney, 1978), the observed differences among them support the notion that they may also play additional roles in the cell. Biochemical experiments suggest that profilin might promote assembly by stimulating the transfer of actin from thymosin β_4 to the barbed filament end (Pantaloni and Carlier, 1993), and possibly by stimulating nucleotide exchange (see above). Indeed, microinjection of profilin-actin complexes into living cells causes an increase in filamentous actin (Cao *et al.*, 1992), while similar injections of cofilin-actin complexes do not (Nagaoka *et al.*, 1995b). The biochemical activities of ADF/cofilins suggest that they may figure more prominently during filament disassembly, and cellular studies indicate that they are are often concentrated at sites of membrane actin cytoskeleton dynamics (Yonezawa *et al.*, 1987; Moon *et al.*, 1993; Saito *et al.*, 1994; Aizawa *et al.*, 1995; Nagaoka *et al.*, 1995a) and can promote filament disassembly and buffering of the monomeric actin that is released. Microinjection of cofilin alone causes a rapid decrease in stress fibers, with a corresponding increase in cofilin-actin rod-like structures (Nagaoka *et al.*, 1995b). These cofilin-actin bars are presumably not composed of normal filaments, as they do not stain with fluorescent phalloidin, and are similar to those formed when cells are subjected to heat shock, 10% dimethyl sulfoxide treatment, or changes in ionic environment (Nishida *et al.*, 1987). Interestingly, some yeast actin mutants also form similar bars (Novick and Botstein, 1985). The function, if any, of these bars is unknown. It is possible that the bars are a "sink" that actin is sequestered into when it is not incorporated into normal cellular structures. Although sequestering proteins such as profilin, thymosin β_4, and ADF/cofilin may control monomer availability in most circumstances, perhaps they cannot effectively buffer actin in times of stress, and bar formation aided by ADF/cofilin ensues as an additional sequestering mechanism.

INTERACTIONS WITH FILAMENTOUS ACTIN

The name "cofilin" stands for "cofilamentous protein" and reflects the filament-binding activity that, upon initial discovery, was more obvious than the depolymerizing activity (Nishida *et al.*, 1984b). When studied in more detail, cofilin was found to possess a pH-sensitive depolymerizing activity (Yonezawa *et al.*, 1985). At pH between 6.3 and 7.3, cofilin cosediments with F-actin and depolymerizes actin to only a limited extent, even when cofilin:actin molar ratios are 2:1. At a pH of 7.8, cofilin displays more depolymerizing activity, but still associates with F-actin. At pH over 8.0, cofilin depolymerizes actin stoichiometrically (Yonezawa *et al.*, 1985; Moriyama *et al.*, 1990b, 1992). These pH effects are completely reversible. Although it was originally reported that recombinant destrin containing a 9-amino acid N-terminal extension does not stably associate with filaments at any pH and that its depolymerizing activity is not pH sensitive (Moriyama *et al.*, 1990b), more recent experiments show that ADF/destrin without an N-terminal extension stably associates with filaments at pH 7.0, and that its depolymerization activity is induced at pH 8.0 (Hawkins *et al.*, 1993; Hayden *et al.*, 1993). Furthermore, the binding of ADF/destrin to filaments is cooperative (Hawkins *et al.*, 1993). Yeast cofilin can be pelleted with actin filaments (Moon *et al.*, 1993) and also shows a pH-sensitive depolymerizing activity (Iida *et al.*, 1993), but actophorin does not appear to stably associate with filaments (Cooper *et al.*, 1986).

Although the in vivo relevance of the pH effects on the activities of ADF/cofilins has not been demonstrated, it has been noted that contexts exist under which such regulation might prove important. For example, cytoplasmic alkalization from pH 7.1–7.3 occurs when quiescent fibroblasts are stimulated with growth factors that also induce cell motility (Moolenaar *et al.*, 1983, 1984), and integrin-mediated adhesion also causes elevation of intracellular pH (Ingber *et al.*, 1990; Schwartz *et al.*, 1991). Alternatively, the pH effects may not reflect a response to elevation of intracellular pH, but might reflect a mechanism in which changes in the local environment caused by formation of a protein complex alter the effective pH.

MECHANISM OF FILAMENT SEVERING

Filament severing occurs by the disruption of noncovalent interactions between actin subunits. Severing is thought to be important for regulating filament length and turnover, and for regulating cytoplasmic viscosity, perhaps inducing "gel-to-sol" transitions during cellular locomotion. A severing activity for the ADF/cofilin proteins was first suggested by Mabuchi (1983) when he conducted electron microscopy experiments and saw that depactin decreased the average length of

filaments. The existence of a severing activity was later supported by multiple criteria, including the findings that the ADF/cofilin proteins reduce F-actin viscosity, increase the polymerization rate in the elongation stage of actin assembly, and increase the number of filaments ends available for assembly and disassembly (Nishida *et al.*, 1984a,b; Cooper *et al.*, 1986). Severing activity has now been confirmed by real-time visual observation (Maciver *et al.*, 1991b).

Severing by the ADF/cofilin proteins differs from severing by gelsolin and related proteins in that the severing activity of gelsolin, but not the ADF/cofilin proteins, is Ca^{++}-dependent and that gelsolin, but not ADF/cofilin, caps filament ends after severing. Visual observation of severing by gelsolin and actophorin reveals an additional difference. Actophorin-induced breaks occur preferentially, although not exclusively, at preexisting filament bends (Maciver *et al.*, 1991b). Gelsolin does not favor breakage at preexisting bends, but appears to induce filament bending before breakage (Bearer, 1991). Combining the data from the visual observations with the previous biochemical experiments allows for the construction of a model (Maciver *et al.*, 1991b) that takes into account filament-binding, filament-severing, monomer-binding, and depolymerizing activities of ADF/cofilins (Figure 1). In this model, ADF/cofilins first bind to subunits along the filament. Thermal motion of a filament transiently creates a "bend" that exposes a normally buried contact for cofilin, allowing cofilin to intercalate between subunits that are within one strand of the two start helix (Maciver *et al.*, 1991b). This would cause further filament destabilization. Finally, complete breakage of the filament would occur as the cofilin-actin complex dissociates from the filament. The products of this reaction would be a severed filament and a sequestered ADP·actin monomer. It has been proposed that the gelsolin-type severing proteins have a more active mechanism of severing in which multiple domains bind to a filament and create a weak link (Kwiatkowski *et al.*, 1989; Way *et al.*, 1989).

ADF/cofilin-induced filament breakdown may also be influenced by nucleotide hydrolysis in the filament. As described above, actophorin has been reported to have a higher affinity for ADP·actin monomers than for ATP·actin monomers (Maciver and Weeds, 1994). In addition, 25 mM inorganic phosphate (Pi), which stabilizes filaments composed of ADP·actin, presumably by reforming the ADP-Pi·actin species (Carlier and Pantaloni, 1988), inhibits the filament-severing and depolymerizing activities of actophorin. This might reflect a similar nucleotide preference for interactions with the filament (Maciver *et al.*, 1991b; Maciver and Weeds, 1994). Alternatively, ADP·Pi-filaments might be poor substrates for severing because they are more rigid before Pi is released (Isambert *et al.*, 1995), making introduction of bends less likely.

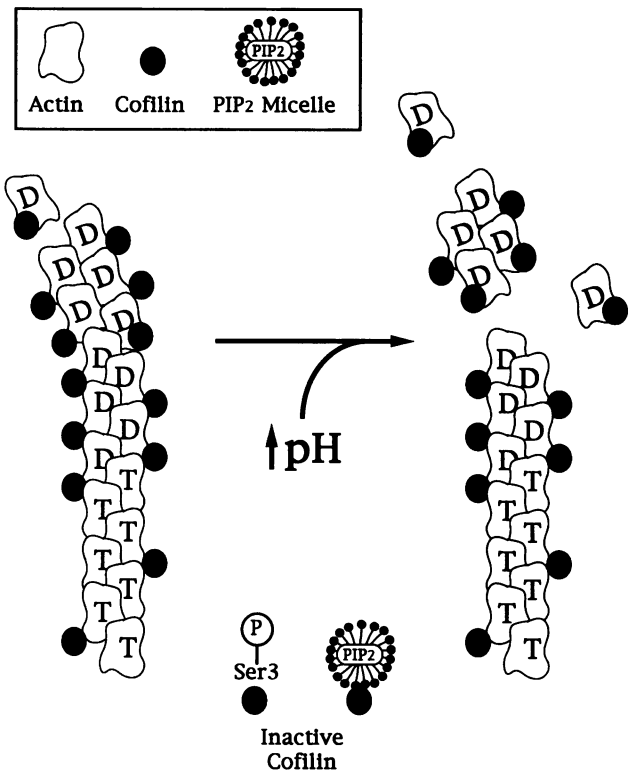

Figure 1. Model of actin filament severing by ADF/cofilins (adapted from Maciver *et al.*, 1991b). ADF/cofilins (represented by black circles) bind to a region on subdomain I of actin subunits in a filament, with greater affinity for monomers that have hydrolyzed their ATP and released the resulting Pi. Bending of a filament reveals additional surfaces for which ADF/cofilins have affinity. The severing protein can now intercalate between subunits, inducing a break in the filament. The actin molecule that was at the site of the bend becomes sequestered. ADF/cofilins may also "nibble" subunits from the ends of filaments via a monomer-sequestering activity. An increase in pH results in the destruction of filaments by ADF/cofilins, most likely by enhancing the severing or both the severing and the sequestering activities. ADF/cofilins that are phosphorylated on Ser3 or are bound to PIP_2 micelles do not interact with actin. (Although the overall severing mechanism is probably conserved among the ADF/cofilins, there appear to be differences in the tendency to stably associate with filaments and in the pH dependence of severing.)

The potential significance of the effects of Pi release on severing is that only older filaments in which ATP has been hydrolyzed and Pi has dissociated would be susceptible to severing by actophorin. This would provide an elegant "clock" mechanism to govern the lifetime of filaments (Maciver *et al.*, 1991b); nucleotide hydrolysis would have dual destabilizing effects, weakening subunit interactions (Pollard, 1986) and increasing the susceptibility to severing. Although the phosphate inhibition of actophorin is specific (at the same concentration, pyrophosphate inhibits actophorin activity more weakly, and sulfate has no effect) (Maciver *et al.*, 1991b), the possibility that phosphate

directly binds to and inhibits actophorin has not been ruled out.

Recent observation of cofilin localization during cytokinesis highlights some important issues concerning function and regulation. Early in the mitotic phase, and even after the cleavage furrow has formed, cofilin is distributed diffusely throughout the cytoplasm. However, during the late stages of cytokinesis when furrowing has progressed, cofilin colocalizes with the contractile ring, and this localization persists until cell division is complete (Nagaoka *et al.*, 1995a). These observations suggest that as cytokinesis proceeds cofilin might mediate the disassembly and possibly the closure of the contractile ring.

How are the localization and function of cofilin regulated? Intrinsic properties of the filament and interactions with other binding proteins might both be important. Cofilin might only bind to contractile ring actin filaments some time after they form, when the actin subunits have hydrolyzed their bound ATP and dissociated the Pi. Interactions with the filament might also be regulated by other filament-binding proteins such as tropomyosin and myosin, which inhibit severing and provide some protection against depolymerization by ADF/cofilin proteins (Bernstein and Bamburg, 1982; Mabuchi, 1982; Nishida *et al.*, 1985). Moreover, the observation that actophorin severs filaments at pre-existing bends suggests the following: 1) filaments in newly formed contractile rings might be poor substrates if Pi has not yet been released since Pi release increases filament flexibility, and 2) filaments under tension in the ring might be poor substrates for severing proteins. The fact that the contractile ring filaments are bundled is also likely to make them resistant to severing as thermal motion of the filaments would be reduced and the probability of reannealing would be high because severed filament ends would not be able to diffuse away from the severed site (Maciver *et al.*, 1991a). Thus, filaments in the contractile ring might only be susceptible to the actions of ADF/cofilins once the filaments have aged and have slid apart (see also Mabuchi, 1986, and the references therein). Additional potential forms of regulation are discussed below.

THE ADF/COFILIN-ACTIN INTERFACE

Understanding how ADF/cofilins sever and why the related proteins coactosin and Abp1p do not sever, and how severing is regulated, depends on a molecular description of the ADF/cofilin-actin interface. Residues in the N-terminal region of depactin can be cross-linked to actin (Sutoh and Mabuchi, 1989) and the extensive homology among the N-termini of ADF/cofilins and studies on ADF phosphorylation further suggest that this region is functionally important (see below). The cofilin region from residues 105–115 has also been cross-linked to actin (Yonezawa *et al.*, 1991) and a synthetic peptide corresponding to this region (^{104}WAPECAPLKSKM^{115}) competes with cofilin for binding to monomeric actin, with its affinity for actin being two orders of magnitude less than that of cofilin. The peptide inhibits actin polymerization but does not bind to F-actin, suggesting that it might bind to an actin surface that is involved in actin-actin contacts in the filament. Mutation of lysines 112 and 114 in this region of cofilin to glutamine abolishes all actin-binding activity (Moriyama *et al.*, 1992), suggesting that the F-actin binding region overlaps this region. There is strong conservation of sequence in this region between the ADF/cofilin proteins of evolutionarily distant organisms (see Table 1). This stretch of residues is also well-conserved in Abp1p, but not in coactosin or drebrin (Table 1).

It has also been suggested that the cofilin sequence ^{122}DAIKKKL^{128} is an actin-binding region, however, the evidence supporting this conclusion is much more tenuous. The impetus for examination of this region derives from homology to a region near the N-terminus of tropomyosin (^{2}DAIKKK^{7}). Tropomyosin competes with cofilin for filament binding (Nishida *et al.*, 1985). When present at a molar concentration one thousand times that of cofilin, the DAIKKKL peptide also inhibits the binding of cofilin to F-actin (Yonezawa *et al.*, 1989). It does not, however, affect the binding of tropomyosin to actin. The DAIKKKL peptide was shown to pellet with actin filaments, but these experiments used extremely high concentrations of actin (26 μM) and peptide (15 mM) and did not include a control peptide to demonstrate specificity. Therefore, the DAIKKKL sequence may not be an important actin contact. The report of x-ray diffraction quality crystals of actophorin provides hope for an atomic model that will be essential to define the interaction surface and to understand the severing mechanism (Magnus *et al.*, 1988; Quirk *et al.*, 1993).

Just as important as defining the actin-binding surface on ADF/cofilins is identification of the complementary surface on the actin filament. The ADF/cofilins can be cross-linked to the N- and C-termini of actin using a zero-length cross-linker (Muneyuki *et al.*, 1985; Sutoh and Mabuchi, 1986), suggesting the binding site for cofilin lies in subdomain 1 of the actin monomer (Kabsch *et al.*, 1990). Some insight into this problem may also be provided by the observation that phalloidin, a mushroom toxin that stabilizes actin filaments (Harwell *et al.*, 1980), inhibits severing by actophorin (Bearer, 1991; Maciver *et al.*, 1991b). Although cofilin and phalloidin compete for binding to filaments (Nishida *et al.*, 1987; Yonezawa *et al.*, 1988), the proposed phalloidin-binding site (Drubin *et al.*, 1993; Lorenz *et al.*, 1993) is far from actin subdomain 1, where cofilin is proposed to bind. Therefore, it is possible that cofilin and phalloidin each induce a con-

formational change in the filament that prevents the other from binding, or that the conclusion that subdomain 1 constitutes part of the ADF/cofilin binding site is in error.

DEPHOSPHORYLATION OF ADF RESTORES ACTIVITY IN VITRO AND CORRELATES WITH CYTOSKELETAL CHANGES IN VIVO

In 1989 it was recognized that cofilin exists in both unphosphorylated and phosphorylated forms in cultured fibroblasts (Ohta *et al.*, 1989). In various tissues and cell lines, 14–61% of total ADF is phosphorylated (Morgan *et al.*, 1993). Early experiments suggested that phosphorylation regulates the cellular compartmentalization of cofilin, because certain cellular stresses (heat shock or dimethyl sulfoxide treatment) caused both dephosphorylation and nuclear translocation of cofilin (Ohta *et al.*, 1989). However, while these two events often correlate (Ohta *et al.*, 1989; Samstag *et al.*, 1994), examples have been found in which dephosphorylation occurs without nuclear translocation (Saito *et al.*, 1994) and in which nuclear translocation occurs without net changes in cellular cofilin phosphorylation levels (Abe *et al.*, 1993), although in the latter case the phosphorylation state of nuclear cofilin was not determined directly. In addition, much of the cofilin found in cells is not phosphorylated but remains cytoplasmic. Therefore, what regulates the nuclear translocation of cofilin, and what function, if any, cofilin serves in the nucleus, remain to be determined.

Evidence now suggests that phosphorylation serves to inhibit ADF/cofilin activity directly. When the activities of unphosphorylated ADF and phosphorylated ADF (pADF) purified from cultured myocytes were compared, it was found that pADF does not sever filaments or induce their depolymerization (Morgan *et al.*, 1993). Treatment of pADF with alkaline phosphatase restores activity (Agnew *et al.*, 1995). Phosphorylation of cofilin and ADF in vivo occurs almost exclusively on serine (Ohta *et al.*, 1989; Morgan *et al.*, 1993; Davidson and Haslam, 1994), and peptide mapping indicated that Ser-3 was the likely site of phosphorylation (Kanamori *et al.*, 1995). Peptide sequencing has confirmed that this residue is phosphorylated in ADF, and, as would be predicted, a Ser3Ala ADF mutant is not phosphorylated in cells (Agnew *et al.*, 1995). The importance of Ser-3 is suggested by the observation that this residue and the following glycine are absolutely conserved between ADF/cofilin proteins in diverse organisms, including yeast and plants (Table 1; see also Agnew *et al.*, 1995). A Ser3Glu ADF mutation intended to mimic the phosphorylated form is an order of magnitude less active in depolymerization assays compared with wild-type ADF (Agnew *et al.*, 1995). These observations suggest that phosphorylation of the amino terminus of ADF/cofilins, a region implicated in the interaction with actin, inhibits filament severing. The next questions concern the function and regulation of ADF/cofilin phosphorylation in vivo.

ADF and cofilin have been identified recently as proteins that undergo rapid dephosphorylation in thrombin-stimulated platelets (Davidson and Haslam, 1994), activated T-cells (Samstag *et al.*, 1994), thyrotropin-stimulated thyroid cells (Saito *et al.*, 1994), and isoproterenol-stimulated parotid glands (Kanamori *et al.*, 1995). These various stimuli all result in changes in cytoskeleton organization and assembly. For example, in thyroid cells, thyrotropin (TSH) induces the macropinocytotic uptake of thyroid hormone precursors. This process involves reorganization of the actin cytoskeleton, including the breakdown of stress fibers, redistribution of actin to the periphery, which now displays substantial ruffling and blebbing, and the formation of pseudopodia that are thought to aid in uptake. Moreover, not only does dephosphorylation of cofilin accompany these morphological changes, but, as discussed above, cofilin is concentrated in regions of the cell cortex active in actin dynamics.

The activation of T-cells, parotid glands, and platelets results in IL-2 secretion, amylase secretion, and dense granule release, respectively. Solation of the cytoplasm is thought to facilitate exocytosis, and the activation of ADF/cofilin would be thought to promote solation. In T-cells and parotid glands the timing of cofilin dephosphorylation is consistent with a role for ADF/cofilin in regulated secretion (Samstag *et al.*, 1994; Kanamori *et al.*, 1995). However, granule release in platelets (more than 60% released after 5 s) precedes the dephosphorylation of cofilin (noticeable only after 15 s), and in this case cofilin activation is better correlated with the cytoskeletal remodeling that leads to morphological changes and aggregation (Davidson and Haslam, 1994).

The signal transduction mechanisms that lead to the activation of platelets, thyroid cells, and T-cells are well-studied phenomena, providing a framework for the investigation of the regulation of cofilin dephosphorylation. In platelets, protein kinase C (PKC) and increased Ca^{++} concentration can synergize to mimic the activation by thrombin. Although PKC activators do not affect the level of cofilin phosphorylation, the Ca^{++} ionophore A23187 causes cofilin dephosphorylation to a similar extent as thrombin (Davidson and Haslam, 1994). In permeabilized platelets, either high Ca^{++}, or GTPγS in the absence of Ca^{++}, causes cofilin dephosphorylation (Davidson and Haslam, 1994). These results suggest that a GTP-binding protein and a Ca^{++}-dependent mechanism play roles in regulating the state of cofilin phosphorylation.

In thyroid cells, both TSH and PKC activation cause rapid disruption of stress fibers and changes in cell morphology, but only TSH induces rapid dephospho-

rylation of cofilin (Saito *et al.*, 1994). TSH is known to act through adenylate cyclase. Therefore, it appears that the PKA, but not the PKC, signaling system controls the rapid dephosphorylation of cofilin in thyroid cells. In astrocytes the cAMP analogue dibutyryl cAMP causes dephosphorylation of ADF and the formation of elongated processes (Baorto *et al.*, 1992), providing another example in which PKA is implicated in dephosphorylation.

Dephosphorylation of ADF/cofilin could result from down-regulation of a kinase or up-regulation of a phosphatase, or both. Neither the phosphatase nor the kinase that regulate ADF/cofilin phosphorylation has been identified. Ser-3 does not fall within a recognized protein kinase consensus site and ADF is a poor substrate for PKC, PKA, myosin light chain kinase, and CaM kinase II in vitro (Morgan *et al.*, 1993). Taken together, the above observations suggest a pathway in which an unidentified kinase inactivates a pool of cofilin by an inhibitory phosphorylation on Ser-3. Extracellular signals are transduced through PKA (possibly regulated by a G-protein-coupled adenylyl cyclase) to activate a phosphatase (or inactivate a kinase) to dephosphorylate cofilin, facilitating the disruption of existing actin networks.

CONCLUSIONS

The ADF/cofilins are endowed with multiple activities that are likely to modulate actin filament dynamics in vivo. Removal of an inhibitory phosphate group from these proteins is tightly coupled to signal transduction pathways that lead to dramatic actin rearrangements in a variety of cell types. Elucidation of roles and regulation of ADF/cofilins is confounded by the presence of multiple other proteins with overlapping biochemical activities, by the complexity of the potential regulatory inputs, and by the difficulty inherent in testing protein functions in living cells. At this time, the most crucial needs are for strategies to test the in vivo roles and regulation of ADF/cofilins and for an atomic model so that specific mechanisms for monomer and filament binding and severing, and for regulation of these interactions, can be formulated. The report of x-ray diffraction quality crystals of actophorin (Quirk *et al.*, 1993), and the identification of ADF/cofilins in each of the eukaryotes with the most powerful genetics (or molecular genetics), mouse (Moriyama *et al.*, 1990a), *Drosophila* (Edwards *et al.*, 1994), *Caenorhabditis elegans* (McKim *et al.*, 1994), *Dictyostelium* (Aizawa *et al.*, 1995), and yeast (Iida *et al.*, 1993; Moon *et al.*, 1993), indicate that these central issues will soon be addressed.

ACKNOWLEDGMENTS

We thank James Bamburg, Sutherland Maciver, Tom Lee, Tom Pollard, and an anonymous reviewer for their help in strengthening this essay. Work in the Drubin laboratory was supported by grants from the National Institute of General Medical Sciences (GM-42759) and the Searle Scholars Program/The Chicago Community Trust.

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